The impact of molecular alterations and the immune microenvironment on the natural history of Follicular Lymphoma including transformation to Diffuse Large B cell Lymphoma

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Abstract

The natural history of follicular lymphoma is heterogeneous with numerous relapses and remissions over many years. A substantial number of patients suffer an aggressive disease course with death due to disease within 5 years of diagnosis. The prognosis is significantly worse in patients (10-68%) who transform to an aggressive histology. The clinical parameters used to stratify patients with this disease have limited discriminative power and new prognostic biomarkers are required. Insights into the biology of the disease, with identification of potential therapeutic targets are also required. Analysis of paraffin embedded diagnostic FL biopsies from populations of patients at the extremes of overall survival (<5 years and >15 years) demonstrated that expression of CD4 T lymphocytes and a perifollicular location of forkhead box protein P3 were significantly more common in diagnostic biopsies from patients who lived >15 years. Patients with high numbers of intrafollicular CD4 T lymphocytes and higher numbers of CD68 positive macrophages were more likely to undergo rapid transformation to diffuse large B cell lymphoma (DLBCL). Analysis of sequential biopsies pre-and post-transformation from patients with FL who subsequently transformed to DLBCL demonstrated high numbers of CD68 positive macrophages in the majority of cases. The overall survival from transformation was reduced in patients in whom the number of FOXP3 positive T cells decreased/remained low compared to patients in whom the number of FOXP3 positive T cells increased/remained high.

Analysis of biopsies pre-and post-transformation from patients with FL who subsequently transformed, identified mutation of *TP53* in 28% of cases suggesting a limited role in the process of transformation. The immunocytochemical expression of MDM2, the *TP53* regulator, was significantly higher on transformation. The phenotype of transformed FL was confirmed immunocytochemically as Germinal Centre type and two potential drug targets, Aurora Kinase B and nm23, were confirmed as being up-regulated on transformation.

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For John Felthouse, Elvira Wisniewska and especially Chris Lee

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List of Abbreviations

APC antigen presenting cell

BCL2 B-cell CLL/lymphoma 2

BCL6 B-cell CLL/lymphoma 6

BCR B cell receptor

BM bone marrow

bp base pair

CD cluster of differentiation

cHD classical Hodgkins disease

CHOP cyclophosphamide, doxorubicin, vincristine and prednisolone

CLL chronic lymphocytic leukaemia

CR complete remission

DAB 3,3'-diaminobenzidene tetrahydrochloride

DLBCL diffuse large B cell lymphoma

EM Electron microscopy

FDC Follicular dendritic cell

FL follicular lymphoma

FLIPI follicular lymphoma international prognostic index

FOXP3 forkhead box protein P3

GC germinal centre

GPR good partial remission

H&E haematoxylin and eosin stain

HRP horseradish peroxidase

ICC immunocytochemistry

IF immunofluorescence

IFN-γ interferon gamma

Ig immunoglobulin

IgH immunoglobulin heavy chain

IL-2 interleukin-2

IL-4 interleukin-4

IL-10 interleukin-10

LN lymph node

MHC major histocompatibility complex

mRNA messenger RNA

NHL non-Hodgkin’s lymphoma

OM original magnification

PBMC peripheral blood mononuclear cells

RAG-1 Recombination activating protein-1

RAG-2 Recombination activating protein-2

TCR T cell receptor

TGF-β transforming growth factor beta

TMA tissue microarray

Treg regulatory T lymphocyte

WHO World Health Organisation

CHAPTER ONE:

Introduction

1.1 General introduction

Mammalian immunity is a sophisticated system which has evolved to protect the body against pathogens. It is composed of two arms, innate immunity and adaptive immunity. The innate immune system responds in a predetermined fashion whereas the cells of the adaptive immune system, lymphocytes, have the capacity to respond to specific antigens. There are two lymphocyte lineages, B lymphocytes and T lymphocytes. Both lineages originate from the bone marrow; however maturation of T lymphocytes occurs in the thymus. Both mature B and T lymphocytes possess membrane receptors which allow them to recognise antigen. The antigen receptors present on B lymphocytes are immunoglobulin (Ig) which recognise free antigen. T lymphocytes can only recognise antigen when bound in a complex to major histocompatibility complex (MHC) on the surface of antigen presenting cells (APC) (Zinkernagel RM, Doherty PC 1974; Zinkernagel RM, Doherty PC 1975). Broadly, there are two kinds of T lymphocytes, T helper cells and T cytotoxic cells but this is now known to be oversimplistic. The cytotoxic T cells kill cells infected with pathogens and the helper T cells coordinate the immune response.

Lymphoid organs are tissues composed of lymphocytes surrounded by a supportive framework of non-lymphoid cells. It is here that the cells of the immune system mature and interact with pathogens. Lymphoid organs are classed as either central or peripheral. The central organs, the bone marrow and thymus, are the locations where lymphocytes are produced. The peripheral organs are where interaction with pathogen occurs, the adaptive responses are initiated and lymphocytes are maintained. Lymph nodes comprise part of the peripheral lymphoid system and are composed of dense collections of lymphoid tissue. Normally lymph nodes are not palpable but infiltration by malignancy or reaction to immune stimulus causes an increase in their size and allows detection.

The peripheral lymph nodes act to filter lymph and allow interaction of antigens, antigen presenting cells and lymphocytes in order to initiate an immune response.

1.2 Histological Structure

Structurally, the lymphoid areas of lymph nodes are divided into three main areas; the cortex, the paracortex and the medulla. Each area contains distinct populations of

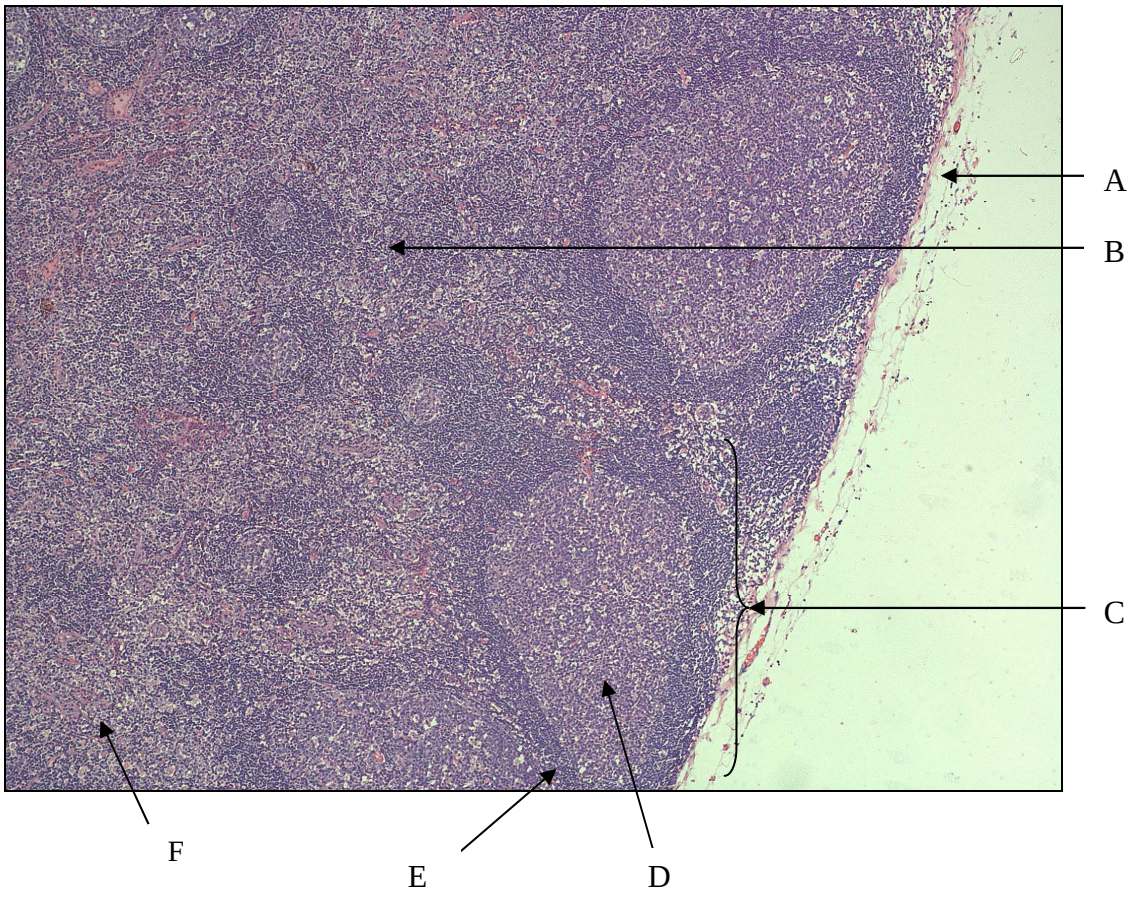
lymphoid cells, which perform different functions. The remainder of the lymph node is composed of fibrous tissue forming a capsule and supporting trabeculae, blood vessels and lymphatic sinuses.

The cortex (superficial cortex) is composed of lymphoid follicles, which form the B cell area of the lymph node and are responsible for humoral immunity. Lymphoid follicles can take the form of either primary or secondary depending on their activation status.

Primary follicles are usually rounded and orientated with their long axis 90° to the capsule of the lymph node. The cells within the primary follicle are inactivated or resting B lymphocytes. Secondary follicles have been activated in response to antigen and are composed of a densely packed mantle area composed of small lymphoid cells surrounding a germinal centre (GC). The germinal centre contains proliferating lymphocytes in all stages of maturation along with stromal cells and antigen presenting cells. The lymphocytes within the germinal centre are predominantly B lymphocytes, which may be small cleaved centrocytes or large centroblasts. Occasional scattered T lymphocytes are present. On activation the germinal centre assumes polarity with a peripheral light area, as seen by haematoxylin and eosin (H&E) staining, composed mainly of centrocytes and a central dark area composed of centroblasts. The centroblasts have a high level of mitotic activity and eventually differentiate into centrocytes (Stevens A, Lowe J 2005). Centroblasts are large cells with vesicular nuclei, prominent nucleoli occupying a peripheral location and a small amount of basophilic cytoplasm (Nathwani BN et al. 2001). On initiation of the immune response those lymphocytes which are the most specific for the antigen are selected to survive and undergo clonal proliferation. Other less specific lymphocytes undergo apoptosis. The surviving centroblasts proliferate and mature into centrocytes which produce immunoglobulins and migrate to the light area of the germinal centre. Some of these cells will become memory B cells; other cells will mature into plasma cells, migrate to the medulla and produce antibody. Thus the reactive germinal centre contains highly mitotic cells juxtaposed with numerous apoptotic cells and apoptotic debris. The mantle zone is composed entirely of B lymphocytes including memory B cells. The cells at the

edges of the mantle zone are less tightly packed and possess more cytoplasm forming a marginal zone (**Figures 1 and 2**).

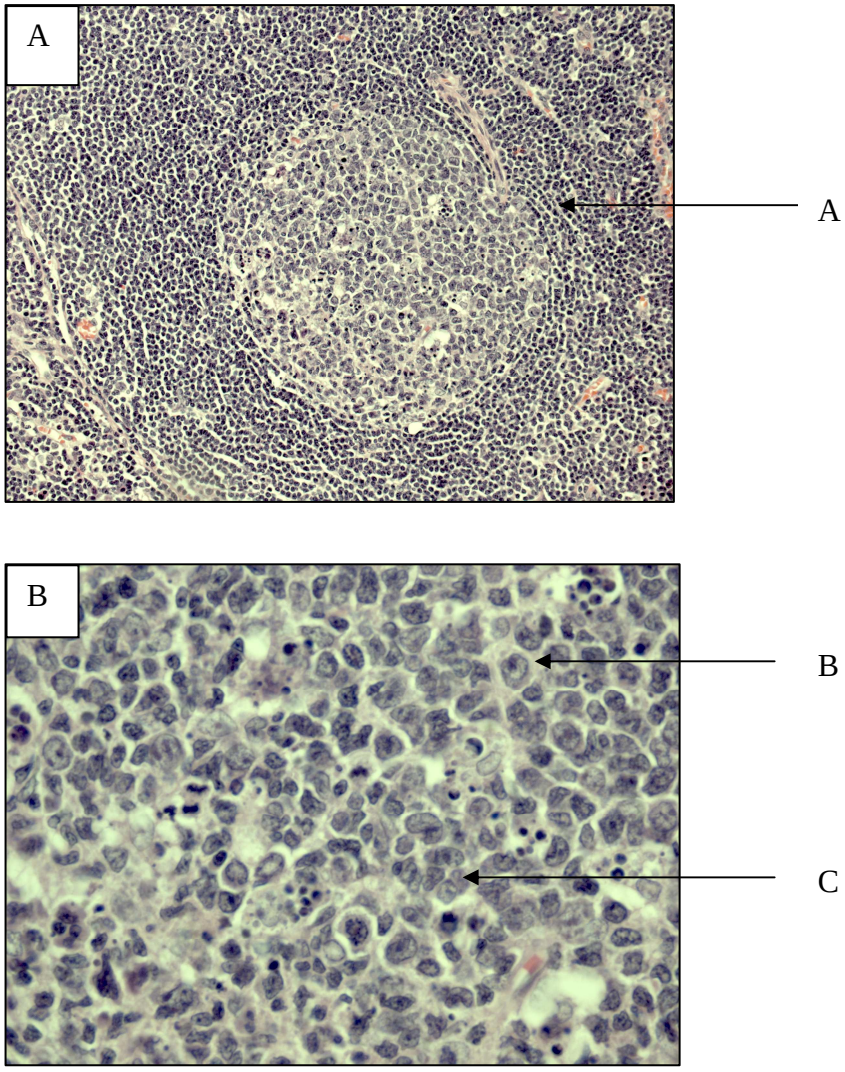
Figure 1 Histological structure of a reactive lymph node stained with haematoxylin and eosin low power photomicrograph original magnification x5



Key:

A) Capsule
B) Primary follicle
C) Secondary follicle
D) Germinal centre
E) Mantle zone
F) Paracortical area

Figure 2 Histology of H&E stained reactive lymph node, A) original magnification x10, B) germinal centre original magnification x40



Key:

A) Mantle zone

B) Centroblast

C) Centrocyte

The paracortical area is the area extending between the lymphoid follicles and contains lymphoid cells, principally T lymphocytes, and venules. The T lymphocytes are of varying sizes as they represent different stages of response to antigen stimulation. Mitotic figures may also be present. Interspersed between the T lymphocytes are interdigitating cells, professional antigen presenting cells, which are essential for the successful initiation of immune response to antigen.

The medulla is composed of cords of lymphoid cells, which form sinuses and extend into the cortex of the lymph node when stimulated by antigen. The medulla represents the area of the node where plasma cell differentiation and proliferation occurs. Admixed with the plasma cells are numerous macrophages, monocytes and mast cells.

The capsule of the lymph node and trabeculae are mainly composed of fibroblasts along with blood vessels, smooth muscle and nerves. The lymphoid cells are supported by a reticular stroma composed of reticular cells and reticulum fibres.

1.3 B lymphocyte development and antigen expression

B lymphocyte development begins in the bone marrow and the fundamental event is Ig gene rearrangement beginning with Ig heavy chain gene rearrangement on chromosome 14q32. The initial step involves joining of the diversity (D) and joining (J) regions to form the DJ segment and subsequent linkage to the variable (V) segment resulting in a functional heavy chain V-D-J region (Tonegawa S 1983) (**Figure 3**).

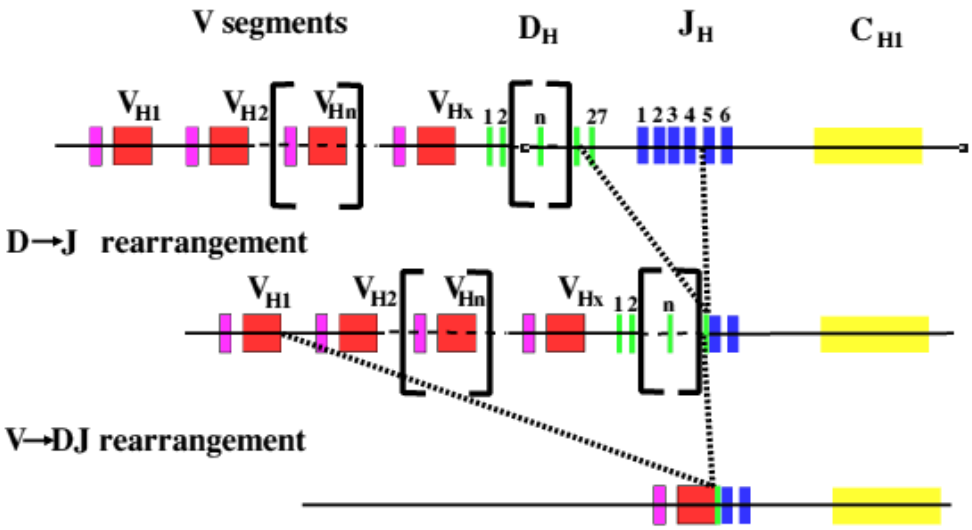


Figure 3 Diagram of VDJ rearrangements in the immunoglobulin heavy chain gene. (Reproduced with permission from N Holmes Cambridge University)

The successful rearrangement of the Ig heavy chain results in suppression of the other copy by allelic exclusion and initiates the rearrangement of the light chain loci. There are two light chain loci, kappa (κ) and lambda (λ). Initially, rearrangement of the κ light chain locus on 2p12 is begun but if unsuccessful the λ light chain locus on 22q11 is rearranged. Cells producing κ light chains have been demonstrated to have unrearranged λ chains whereas those producing λ light chains have rearranged and inactivated κ chains (Hieter et al. 1981, Korsmeyer SJ et al. 1982). The process of recombination is performed by recombination activating gene-1 (RAG-1) (Schatz DG, Oettinger MA, Baltimore D 1989) and recombination activating gene-2 (RAG-2) (Oettinger MA et al. 1990). If functional heavy and light chains are not produced, the cell undergoes apoptosis within the bone marrow. Surviving cells mature to form naïve B cells expressing IgM and IgD as a result of alternative splicing of the constant region genes. Encounter with specific antigen causes naïve B cells to proliferate and stop production of IgD. This proliferation occurs in the GC of reactive lymph nodes and some of these proliferating cells will produce IgM. Within GC the B cell blasts undergo somatic hypermutation of the V region resulting in some cells with increased affinity for the antigen. These cells preferentially expand and any cells with a lower affinity to the antigen undergo apoptosis. This process is

known as affinity maturation and produces the secondary antibody response (Griffiths GM et al. 1984; Paul WE et al. 1967). Activation by antigen causes class switching to occur allowing production of antibodies of isotypes IgG, IgA or IgE. The heavy chain constant region genes are situated at the 3' end of the locus and ordered from 5' to 3'; μ,δ,γ,α,ε. Initially IgM is the only functionally Ig transcript but eventually a specific constant region is selected and the others deleted (Andersson J, Coutinho A, Melchers F 1978; Gearhart PJ, Hurwitz JL, Cebra JJ 1980).

The stages of normal B lymphocyte development are fundamental to understanding the classification of B cell lymphomas (**Table 1**). The earliest B lineage committed cell is the progenitor B cell (pro-B). These are blast cells which undergo Ig gene rearrangement. Once gene rearrangement has occurred and cytoplasmic μ is expressed the cell is known as a pre-B cell and light chain gene rearrangement occurs with eventual surface expression of IgM. Both pro and pre-B cells express terminal deoxynucleotidyl transferase (TdT) a DNA polymerase which helps add to diversity of the lymphocyte antigen recognition sites by addition of nucleotides. The cell is now an immature B cell. These cells circulate in the bloodstream and form primary follicles and mantle zones in the primary and secondary lymphoid organs. Naïve mature B cells undergo blastic transformation on encountering antigen and proliferate. These B blasts then migrate into the centre of primary lymphoid follicles and form a germinal centre (Liu YJ et al. 1991). The blast cells are called centroblasts and often down-regulate Bcl-2 protein increasing their susceptibility to apoptosis (Hockenbery DM et al. 1991; McDonnell TJ et al. 1989; Nunez J et al. 1990). Centroblasts will become centrocytes, cells which express surface Ig but may have undergone class switching and somatic mutation. The cells with high affinity for the antigen are rescued from apoptosis by the process of binding to antigen in the processes of follicular dendritic cells (FDCs). These cells up-regulate Bcl-2 protein (Liu YJ et al. 1991). Bcl-6 expression is down-regulated as a result of interaction with CD23 on follicular dendritic cells (FDCs) and CD40 ligand (CD40L) on T lymphocytes (Cattoretti G et al. 1995; Pittaluga S et al. 1996). B cell lymphocyte development is a complex and highly organized process. The stages of differentiation can be assessed by expression of cell surface proteins which can be recognized by monoclonal antibodies.

Table 1 B lymphocytes and neoplastic equivalents

B CELLS	SURFACE MARKERS	NEOPLASTIC EQUIVALENT
Pro B cell	Tdt, CD19, CD22, CD34	Lymphoblastic lymphoma (B cells)
Pre B cell	Tdt, CD9, CD19, CD34, CD10	Lymphoblastic lymphoma (B cells)
Immature B cell	CD19, CD20, CD10, Ig	Lymphoblastic lymphoma (B cells)
Mature naïve B cell	CD19, CD20, CD23, CD5	Small lymphocytic lymphoma, Mantle cell lymphoma
Germinal centre cell	CD10, CD19, CD20, CD23, CD5	Follicular lymphoma, Diffuse large B cell lymphoma, Burkitts lymphoma
Memory B cell	CD19, CD20, CD79a	Marginal zone lymphoma, small lymphocytic lymphoma
Plasma cell	CD38	Plasmacytoma, myeloma

Adapted from Küppers R et al. 1999 and Dey P 2006

All the B lymphocytes present within lymphoid follicles express the pan B cell markers CD19, CD20 and CD22. CD19 is a cell membrane antigen, which is present in all B lymphocytes except plasma cells. Its expression occurs very early in B lymphocyte development and so may be more sensitive than CD20 in identifying lymphoblastic populations (Nadler LM et al. 1983). CD20 is a phosphoprotein, which is expressed on the cell membrane of B lymphocytes (Stashenko P et al. 1980) and the majority of B cell lymphomas (Nadler LM et al. 1981). It is not expressed by pre B cells or plasma cells (Tedder TF et al. 1985). CD22 expression is limited to resting and activated B lymphocytes and is a two chain glycoprotein. Its cytoplasmic expression is seen very early in B lymphocyte development and is subsequently detected on the cell membrane. After activation initially expression increases but is subsequently down-regulated. Expression is seen in approximately half of B cell lymphomas (Dörken B et al. 1986). CD22 plays an important role in the early stages of B cell activation and proliferation (Pezzutto A et al. 1987). The centroblasts within the secondary follicles stain positively for CD10 (Harris NL et al. 1984) (**Figure 8**) and Bcl-6 (Cattoretti G et al. 1995) (**Figure 9**). CD10 was originally detected in rabbit kidney brush border and is a neutral endopeptidase (Kerr MA, Kenny AJ 1974) and subsequently was demonstrated to be an antigen in acute lymphoblastic leukaemia; the common acute lymphocytic leukaemia antigen (CALLA) (Greaves MF et al. 1975). CD10 is a cell membrane antigen, which is present on follicular centre B cells (Weinberg DS et al. 1986), granulocytes (Braun MP et al. 1983) and precursor lymphoid cells (Pesando JM et al. 1983). It is a regulator of B cell growth and proliferation (Cutrona G et al. 2002). Bcl-6 is a zinc finger transcription factor, which is expressed in the nucleus of germinal centre B cells but not by plasma cells or immature B cells, suggesting it may play a role in germinal centre development and/or function (Cattoretti G et al. 1995). Bcl-2 protein product when down-regulated makes cells susceptible to apoptotic cell death (Hockenbury DM et al. 1991). The B lymphocytes in the mantle area show positivity for IgD and bcl-2. Centroblasts and centrocytes within reactive follicles are negative for bcl-2 (**Figure 9**). B lymphocytes either express κ or λ immunoglobulin light chains in a ratio of approximately 2:1.

1.4 Other cells in the germinal centre

Many other cells are also present in the germinal centre and these different compartments can be highlighted using immunocytochemistry. Numerous T lymphocytes are present and these are identified by positivity for CD3, part of the T cell receptor complex. T cell receptor mRNA was identified as specific for T lymphoblasts, thymocytes and phytohaemagglutinin activated T lymphocytes and was demonstrated to have similarity to both the immunoglobulin light and heavy chains (Yanagi Y et al. 1984). A monoclonal antibody was generated against cell surface determinants of T lymphocytes and OKT3 was demonstrated to react against all human peripheral T lymphocytes (Kung P et al. 1979). Antisera raised against CD3 antigen (Mason DY et al. 1988) and subsequently monoclonal antibodies generated against CD3 peptide were demonstrated to be able to detect both reactive and neoplastic T lymphocytes in paraffin embedded tissue (Mason DY et al. 1989) (**Figure 10**).

The CD5 antigen is a 65,000-dalton glycoprotein present on the surface of both mature and immature T lymphocytes (Royston I et al. 1979). Although initially considered to be expressed only on T lymphocytes it has subsequentially been found some B cell malignancies including chronic lymphocytic leukaemia (CLL) (Royston I et al. 1980) and on subsets of B lymphocytes (Caligaris-Cappio F 1982). Traditionally expression of CD5 assessed by immunocytochemistry has been used to indicate T lymphocytes as its membrane expression is approximately 30-fold higher on T lymphocytes than B lymphocytes (Kaplan D et al. 2001).

The monoclonal antibody Ki67 detects a human nuclear antigen present in proliferating cells but not in quiescent cells (Gerdes J et al. 1983). The nuclear antigen is present in all stages of the cell cycle except G0 and as such is a reliable way to evaluate the proliferation fraction of both reactive and neoplastic tissues (Gerdes J et al. 1984) (**Figure 11**).

The presence of activated B lymphocytes within a primary follicle in lymph nodes results in the formation of a germinal centre containing a follicular dendritic cell (FDC) meshwork. FDCs capture antigen as immune complexes of antigen, antibody and complement and then display, but do not classically present, antigen to B

lymphocytes. In vitro data demonstrates that FDCs, in association with T lymphocytes, are essential for adhesion and proliferation of B lymphocytes. The presence of FDCs induces the B lymphocytes to form large clusters and lymphocytes, in contact with FDCs, were resistant to apoptosis (Kosco MH, Pflugfelder E, Gray D 1992). Subsequently B lymphocytes are activated so they can act as antigen presenting cells for T lymphocytes (Kosco-Vilbois MH, Gray D, Scheidegger D, Julius M 1993). The presence of complement receptors on B lymphocytes was initially described over thirty years ago (Lay WH, Nussenzweig 1968; Bianco C, Patrick R, Nussenzweig 1970) resulting in the hypothesis that there was an interaction between complement and the humoral immune system. Two distinct complement receptors are present on the surface of all mature B lymphocytes; CR1 (CD35) and CR2 (CD21) (Tedder TF, Fearon DT, Gartland GL, Cooper MD 1983; Tedder TF, Clement LT, Cooper MD 1984). CD21 is, in addition, the receptor for Epstein Barr virus (EBV) on B lymphocytes (Fingeroth JD et al. 1984). CD21 is associated with a complex of membrane proteins including CD19 which act as signal transducers once CD21 is bound (Matsumoto AK et al. 1991). There are two types of B lymphocytes; B-1 cells which express CD5 and are self renewing, producing naïve B cells in lymphoid tissue and B-2 cells which are CD5 negative and are renewed in the bone marrow. Both cells respond to antigen; however the B-1 cells have a more limited antigen recognition repertoire. A murine model with deregulation of *Cr2* locus demonstrated a reduction in the numbers of B-1 cells in the peritoneum coupled with a defective humoral immune response to T lymphocyte dependent antigens. The reduced humoral response was evidenced by reduced numbers and size of germinal centres in the spleen and lowered serum antibody levels. Bone marrow transplantation from *Cr2* normal donors restored humoral immunity to normal (Ahearn JM et al. 1996). This result postulates a role for CD21 in antibody response. Further murine models deficient in both CR1 and CR2 demonstrated normal B and T lymphocyte development but an impaired response to both low and high doses of T cell dependent antigen. The activation of B lymphocytes is not completely abolished as there is still generation of germinal centres and some secondary antibody response (Molina H et al. 1996). The situation is a little less clear in humans than mice.

CD23, also known as FcεRII, is a C type lectin which is the low affinity receptor for

immunoglobulin E (IgE). FDCs were isolated from tonsils and reactive lymph nodes and the presence of CD23 detected on 50% of the FDC population. FDCs isolated from low grade non-Hodgkin lymphoma also expressed the same antigenic profile including CD23 indicating that FDCs have the same antigenic profile in both reactive and neoplastic lymphoid tissue (Petrasch S et al. 1990). Supernatants containing soluble CD23 facilitate the survival of germinal centre B lymphocytes as well as encouraging differentiation towards plasma cell morphology. These effects were reduced after soluble CD23 was removed from the supernatant. The combination of recombinant CD23 with interleukin 1 alpha produced rescue and differentiation of germinal centre cells (Liu YJ et al. 1991). CD21 has been demonstrated to act as a ligand for CD23 and the combination of these molecules increases interleukin-4 induced IgE production by mononuclear cells (Aubry JP et al. 1992).

CD21 and CD23 are also expressed on other cells and immunocytochemistry for CD21 and CD23 demonstrates the presence of a follicular dendritic cell meshwork in lymphoid tissue (**Figure 10**).

1.5 Principles of Immunocytochemistry

Immunocytochemistry is a technique which uses antigen-antibody interaction to demonstrate the presence of an antigen on a cell. The location of the antigen binding is demonstrated either by direct labeling of the antibody or the use of a secondary antibody labeling system. As mentioned, there are two techniques for visualization, direct and indirect. The traditional direct method is simple as it requires only one reagent with subsequent use of the appropriate chromogen substrate. This technique most commonly has a fluorochrome as a conjugate however this would be unsuitable for use with tissue when morphological interpretation is required and horseradish peroxidase (Nakane P, Pierce GB 1966) or alkaline phopshatase are used instead (**Figure 4**). The indirect method is more complex as a two step process is required, the primary antibody is applied initially and a secondary labeled antibody directed against the first is then applied (**Figure 5**). Usually the label is horseradish peroxidase and this is used in combination with a chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB). This results in an insoluble, stable brown end-product (Graham RC, Karnovsky MJ 1966). This technique is slightly more complicated than the direct method but is more sensitive.

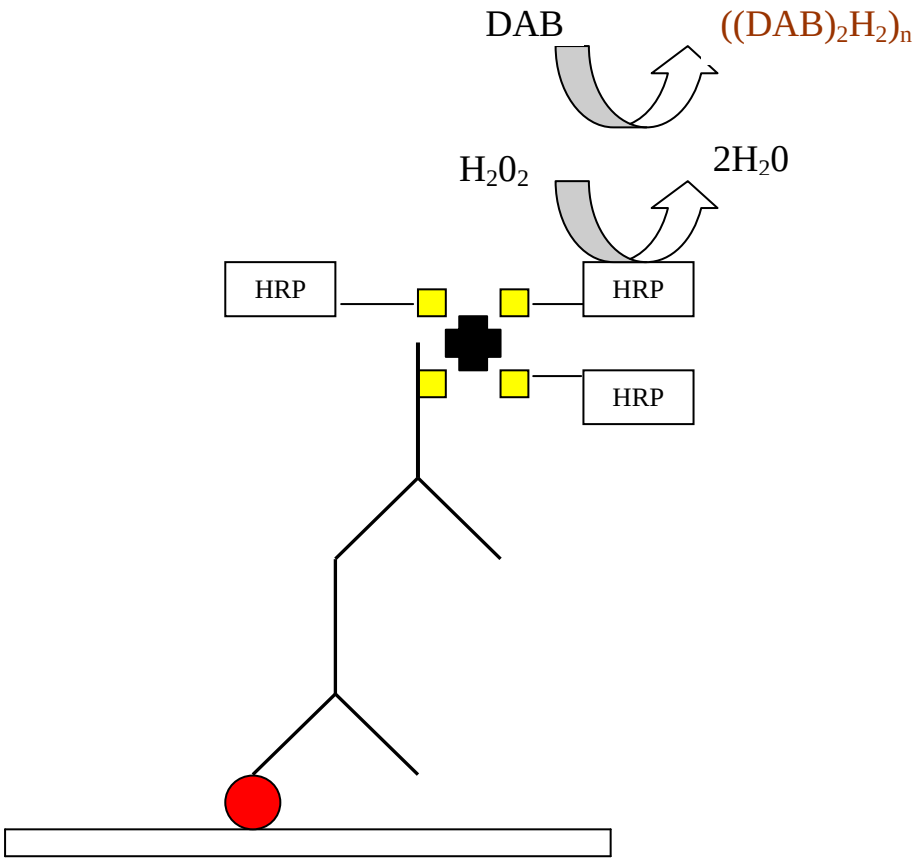
Figure 4 Direct method of immunocytochemistry using peroxidase (P) conjugated anti-human antibody

Figure 5 Indirect method if immunocytochemistry using a human antibody e.g. from a rabbit and a secondary peroxidase (P) conjugated antibody against rabbit

Antigen

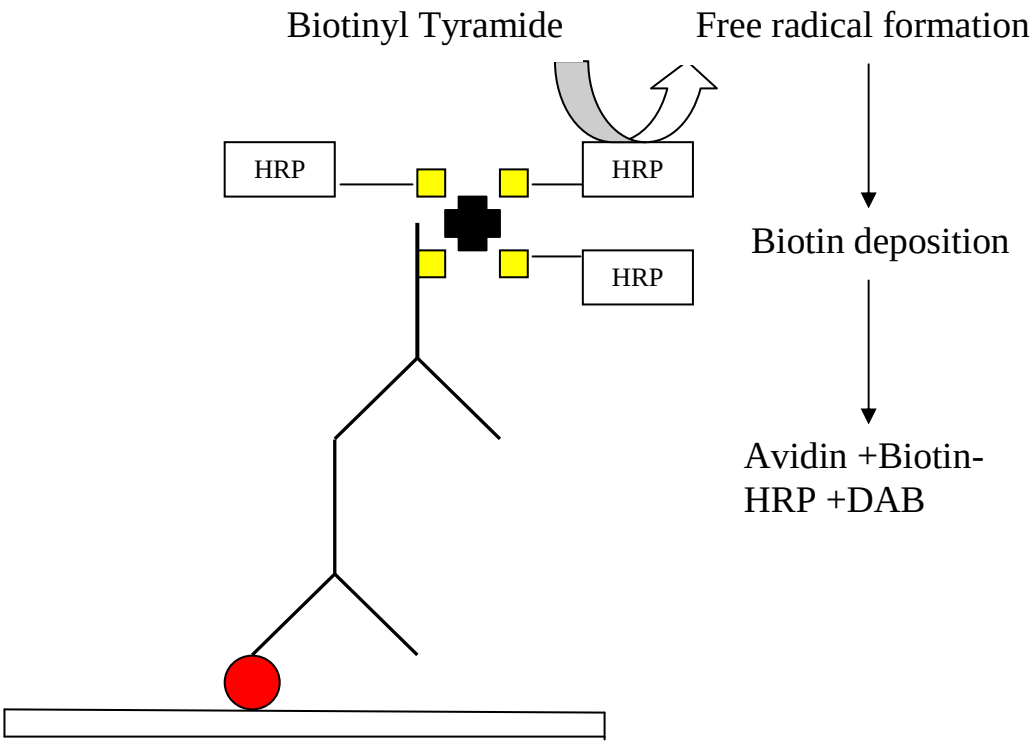
The use of avidin-biotin techniques increased sensitivity of antigen detection. Avidin has four binding sites for biotin. Biotin is easily conjugated to antibodies and enzyme markers. The secondary antibody is biotinylated and binds to one of the four biotin binding sites on the avidin. Peroxidase conjugated biotin is then added and binds to the other three biotin binding sites on the avidin (Hsu SM, Raine L, Fanger H 1981) (**Figure 6**).

Figure 6 Diagrammatic representation of the use of indirect antibody immunocytochemistry with the avidin (black) and biotin (yellow) technique. HRP = horseradish peroxidase DAB= 3,3'-diaminobenzidine tetrahydrochloride



Although the avidin biotin methodology is routinely used in many laboratories there are some antigens which require amplification methods in order to be visualized. The tyramide amplification technique relies on the ability of phenolic compounds to become highly reactive and unstable when oxidized (Gross AJ, Sizer IW 1959). Dimerization of proteins can occur when biotinyl tyramide is oxidized (Bobrow MN et al. 1989) and this is used in immunocytochemistry to generate reactive biotinyl-tyramide intermediates which bind to the protein close to peroxidase enzymes. This results in numerous biotin signals. Usually the primary antibody is associated with the peroxidase enzymes in a routine manner (avidin-biotin complex), the biotinyl tyramide and hydrogen peroxide are applied and generate biotin which is used to capture the peroxidase enzymes (Adams JC 1992) (**Figure 7**).

Figure 7 Diagrammatic representation of the use of tyramide amplification immunocytochemistry with the avidin (black) and biotin (yellow) technique. HRP = horseradish peroxidase DAB= 3,3'-diaminobenzidine tetrahydrochloride



Tissue used for routine diagnostic histology must be preserved in a fixative, typically formalin in the United Kingdom. The material is then processed and embedded in paraffin wax prior to sectioning. Cross-linking by the formalin results in masking of epitopes of the antigen (Mason JT, O'Leary 1991) and consequently retrieval of the antigen must be performed before immunocytochemistry can be performed successfully. Typically antigen retrieval is performed in either of the following ways; pressure cooking antigen retrieval or proteolytic enzyme digestion. Pressure cooking antigen retrieval boils the dewaxed paraffin sections in solutions (usually 0.01M citrate buffer (pH 6.0) although this varies with differing antigens) (Norton AJ, Jordan S, Yeomans P 1994). The enzyme digestion retrieval technique typically uses trypsin or protease (Huang S et al. 1976, Curran RC, Gregory J 1977). The exact mechanisms whereby enzymatic digestion releases the antigens is unknown, however it is presumed that digestion breaks down formalin crosslinking.

Figure 8 Immunocytochemical phenotype of the germinal centre in a reactive lymph node, A) CD10 showing positive staining in GC original magnification x10 B) CD20 original magnification x10

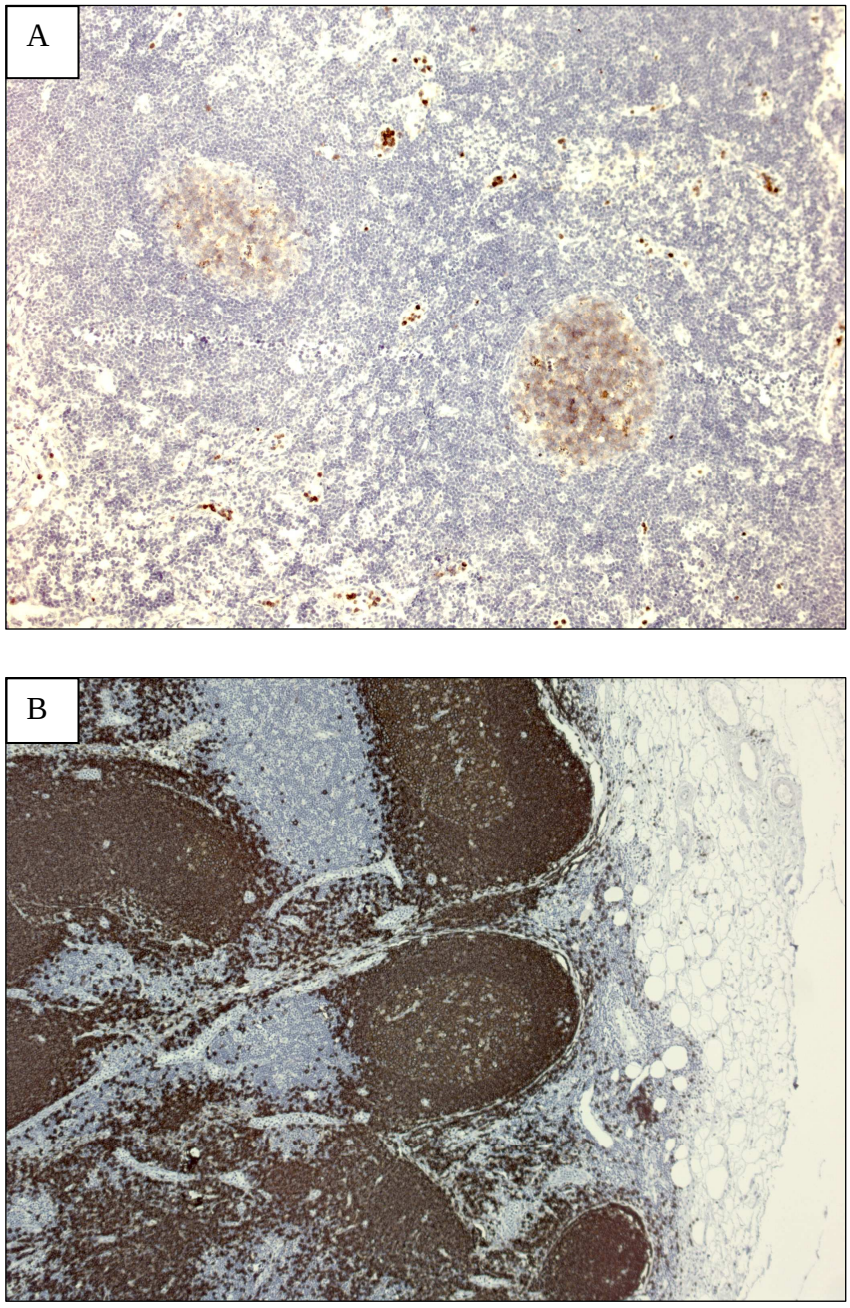


Figure 9 Immunocytochemical phenotype of the germinal centre of a reactive lymph nodes, A) Bcl-2 demonstrating negative staining of the germinal centre in a reactive follicle original magnification x10, B) Bcl-6 demonstrating positive staining of cells within the germinal centre.

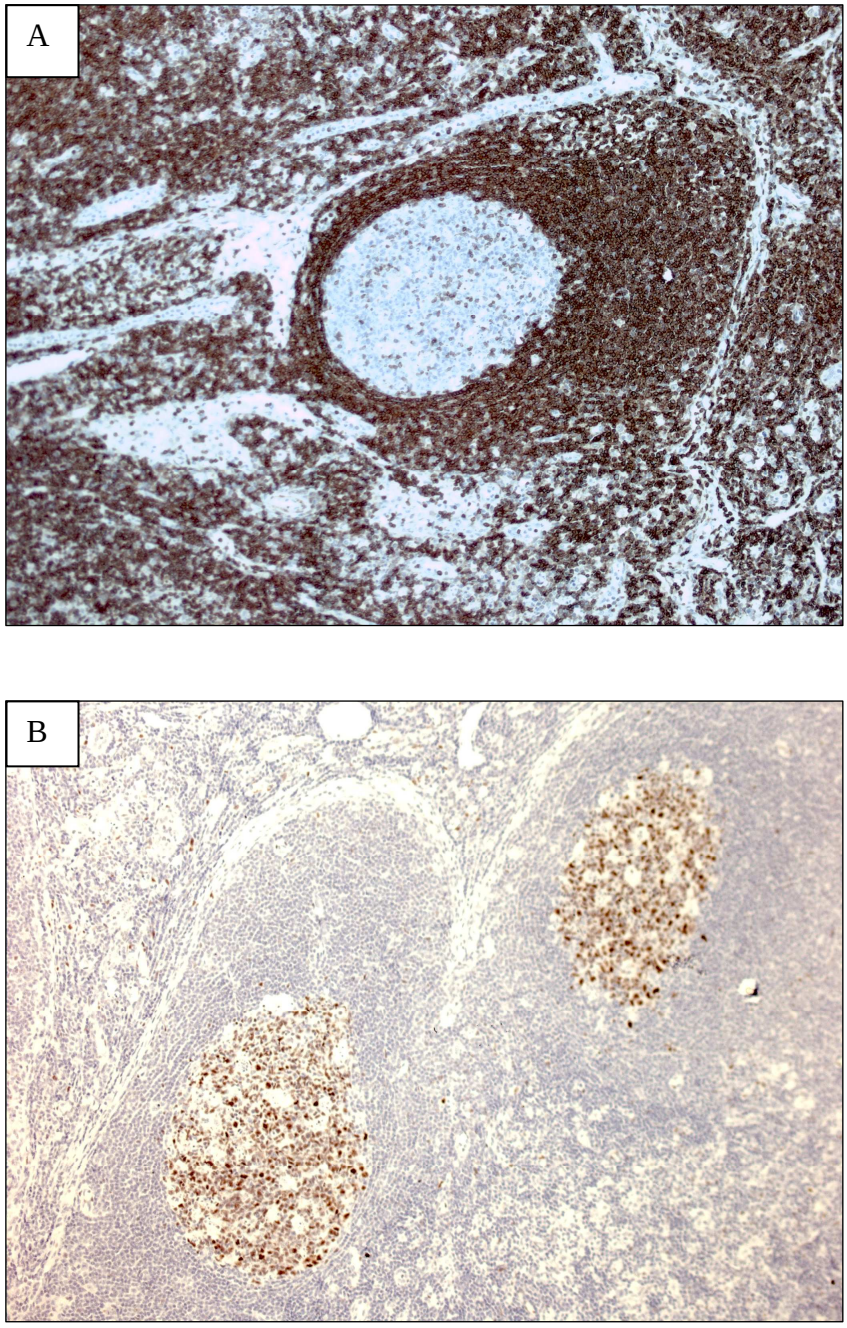


Figure 10 Immunocytochemistry of the other cells present in the reactive lymph node, A) CD3 demonstrating reactive T lymphocytes mainly surrounding the reactive follicles but some within the follicles original magnification x10, B)CD21 demonstrating follicular dendritic meshwork in the follicles original magnification x10, C) CD23 demonstrating follicular dendritic meshwork within the follicles original magnification x10

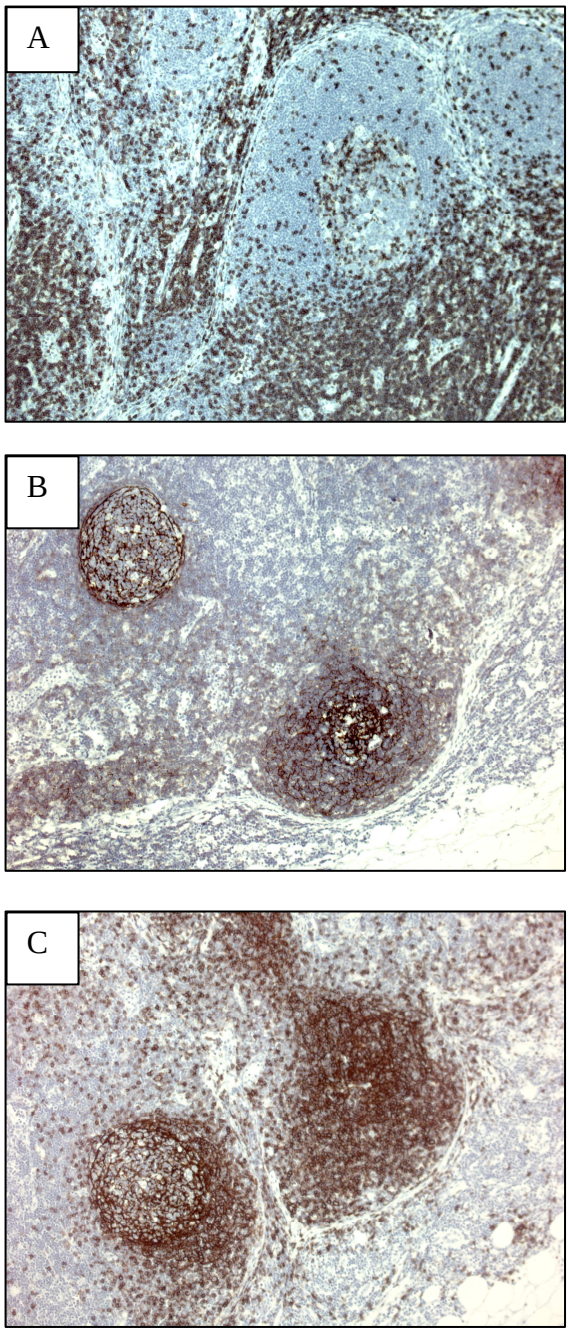
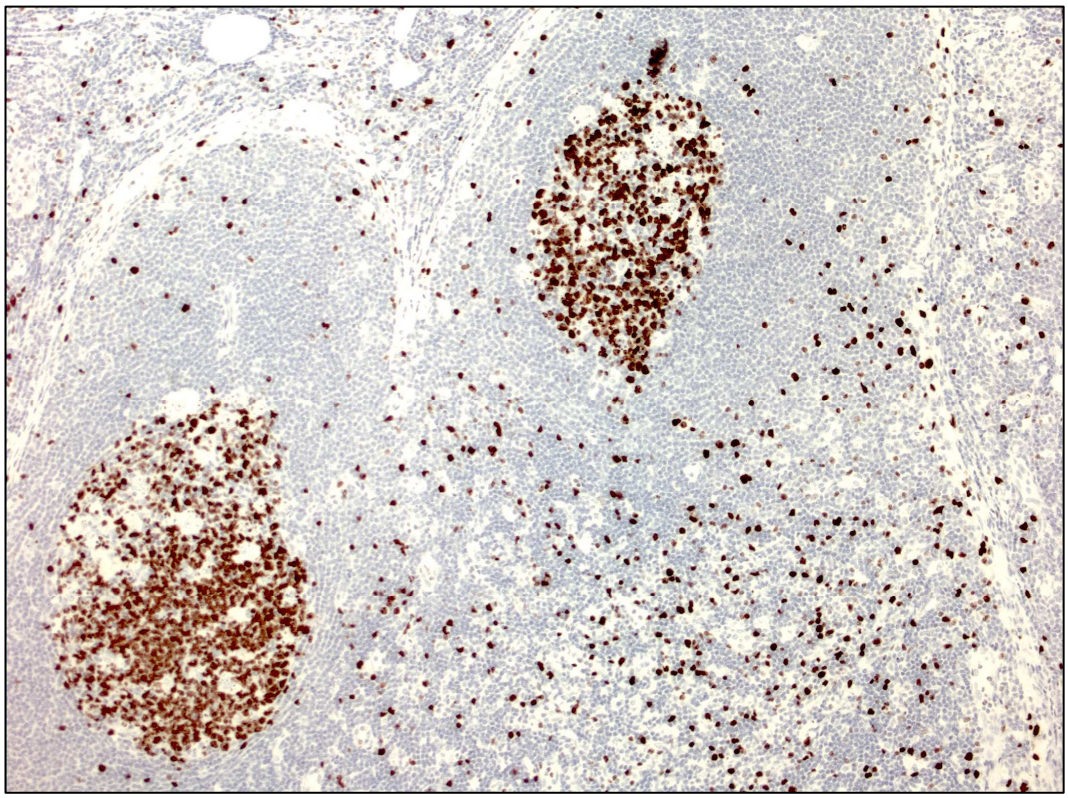


Figure 11 Immunocytochemistry for the proliferation marker Ki67 within the germinal centre of a reactive lymph node demonstrating the presence of numerous cells in cell cycle original magnification x10



1.6 Mature B cell neoplasms

Mature B cell neoplasms comprise more than 90% of all lymphoid neoplasms worldwide (Anon 1997; Armitage JO, Weisenberger DD 1998) and are more common in developed countries. They represent clonal proliferations of B cells in all stages of differentiation and the classification of these neoplasms is based to some extent upon this phenomenon.

Lymphomas are classified currently using the World Health Organization Classification of tumours system which was published in 2001 and has been updated in 2008. This system has evolved from a myriad of previous classification schemes and is based on the morphology, immunophenotype, genetics and clinical features of the disease.

The most common are follicular lymphoma (FL) and diffuse large B cell lymphoma (DLBCL) which account for approximately half of all non-Hodgkin's lymphomas (NHL) (Anon 1997; Armitage JO, Weisenberger DD 1998) and myeloma.

1.7 Follicular lymphoma

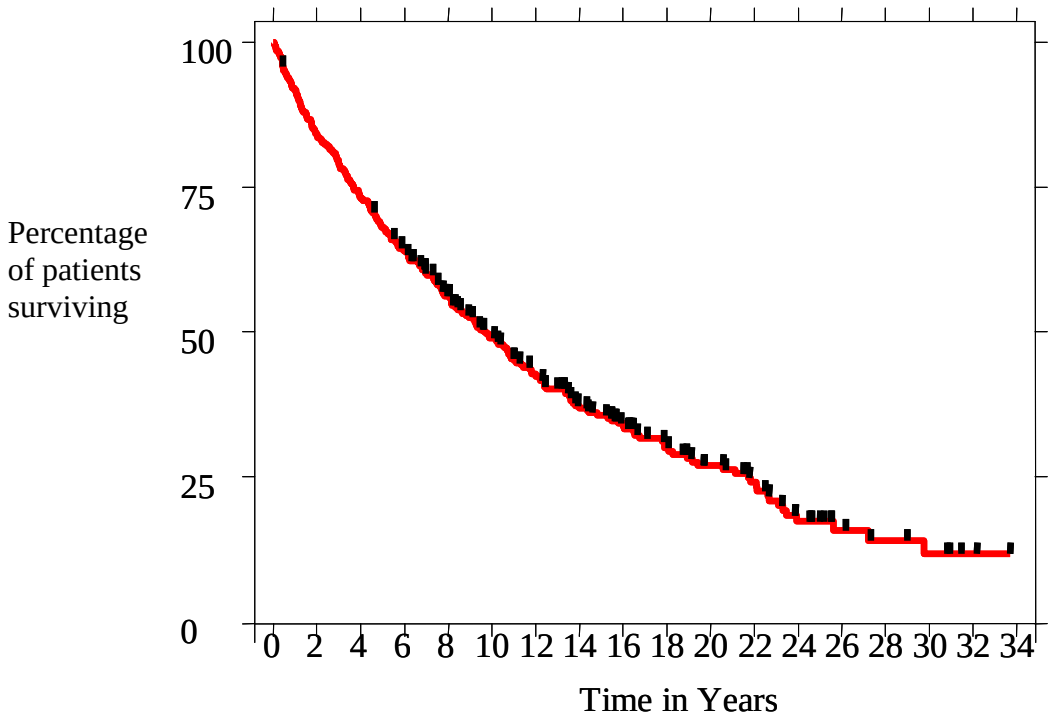
Follicular lymphoma is a malignant neoplasm of the follicle centre cells (centrocytes and centroblasts) typically with a characteristic follicular pattern of growth. It is the most common of the 'indolent' NHLs and accounts for 35% of adult NHL in the United States and 22% worldwide. Follicular lymphoma was first described in 1925 by Brill, Baehr and Rosenthal who observed a clinical and pathological disease which presented with lymph follicle hyperplasia in the spleen and lymph nodes (Brill NE et al. 1925). Subsequently, Baehr and Rosenthal suggested that the disease was a tumour of lymph nodes arising in a multifocal pattern (Baehr G; Rosenthal N 1927). The possibility of transformation of follicular lymphoma to other lymphoid malignancies as part of the natural history of the disease was first observed and published in 1938 (Symmers D 1938).

1.7.1 Clinical features

Follicular lymphoma is a disease seen mainly in adults with a median age at presentation of 59 years and a slight female preponderance (Anon 1997). Commonly affecting the peripheral lymph nodes it also can involve the spleen, bone marrow and

peripheral blood. In patients with widespread disease numerous extra-lymphoid sites including the gastrointestinal tract may be affected. Patients typically present with late stage widespread disease however they are usually asymptomatic. The median survival is ten years however there is a wide variation in overall survival with some patients dying from disease very quickly after diagnosis and others surviving for several decades (**Figure 12**). Death occurs as a result of disease progression in just under half of all cases (46%) with another 17% dying as a consequence of treatment related complications (Montoto S et al. 2007).

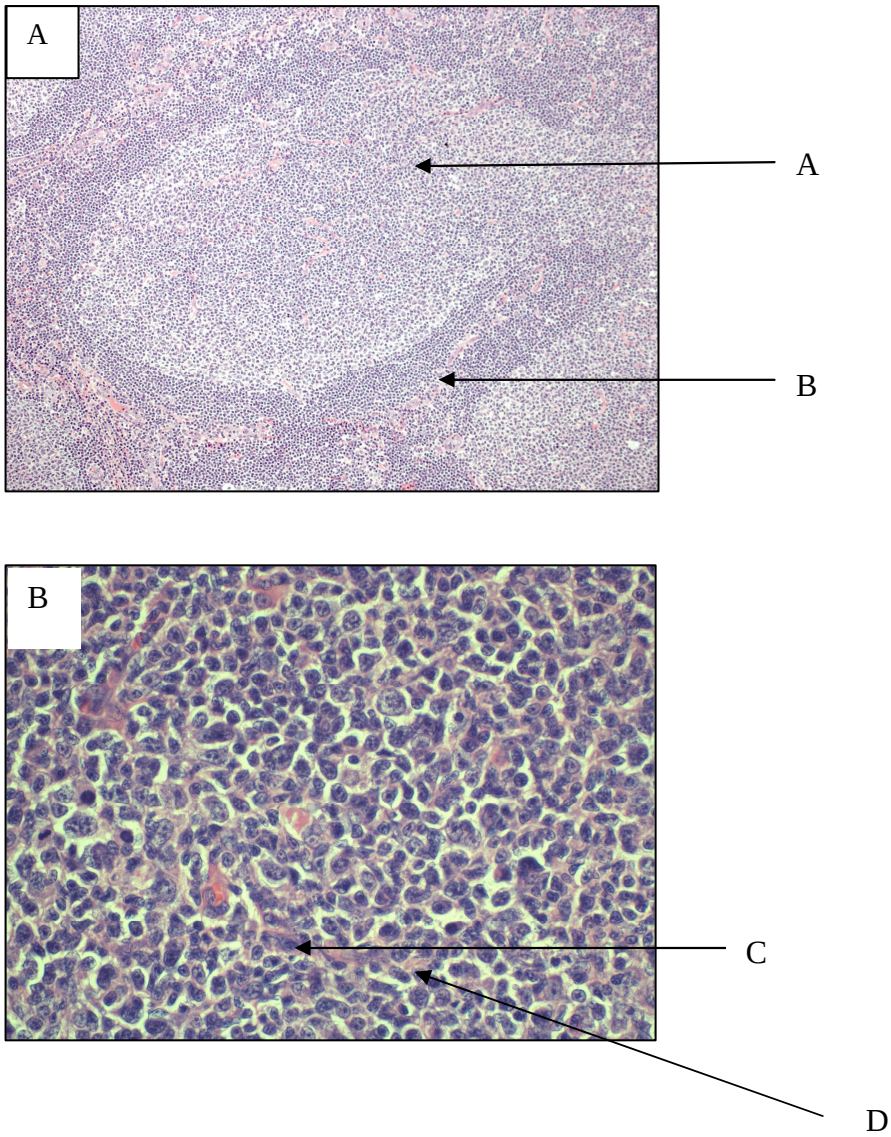
Figure 12 Overall survival from diagnosis of patients diagnosed with follicular lymphoma at St Bartholomew's Hospital (Montoto S et al. 2005; 2007)



1.7.2 Histological structure and grading

In keeping with the nomenclature and cell of origin the majority of follicular lymphomas are composed of neoplastic follicles with a follicular growth pattern. In comparison to normal reactive follicles the neoplastic follicles do not possess mantle zones or show polarization and are ill defined and closely packed. Neoplastic cells are also present in the areas between the follicles. There is usually complete effacement of architecture with extension of neoplastic cells over the capsule and sclerosis is often observed. Additionally loss of tingible body macrophages is seen along with a reduction in the number of apoptotic bodies. Within the follicles the neoplastic cells comprise both centrocytes and centroblasts (**Figure 13**). Recently an additional morphological pattern has been described with focal germinal centres staining positively for bcl-2 protein with a reactive morphology and immunophenotype in the remainder of the lymph node. These bcl-2 positive follicles have been shown to express monoclonal IgH gene rearrangements. In some patients there was synchronous FL at a different site and other patients subsequently developed FL. This pattern has been termed ‘in situ’ follicular lymphoma and may represent an early development stage of FL (Cong P et al. 2002).

Figure 13 Morphology of follicular lymphoma stained with H&E, A) Low power photomicrograph of a neoplastic follicle original magnification x10, B) Photomicrograph of a neoplastic follicle original magnification x 40



Key:

A) Neoplastic follicle

B) Residual mantle zone

C) Centroblast

D) Centrocyte

Centrocytes are small to medium in size and contain cleaved or twisted nuclei. Centroblasts are large cells with a small rim of basophilic cytoplasm, vesicular nuclei

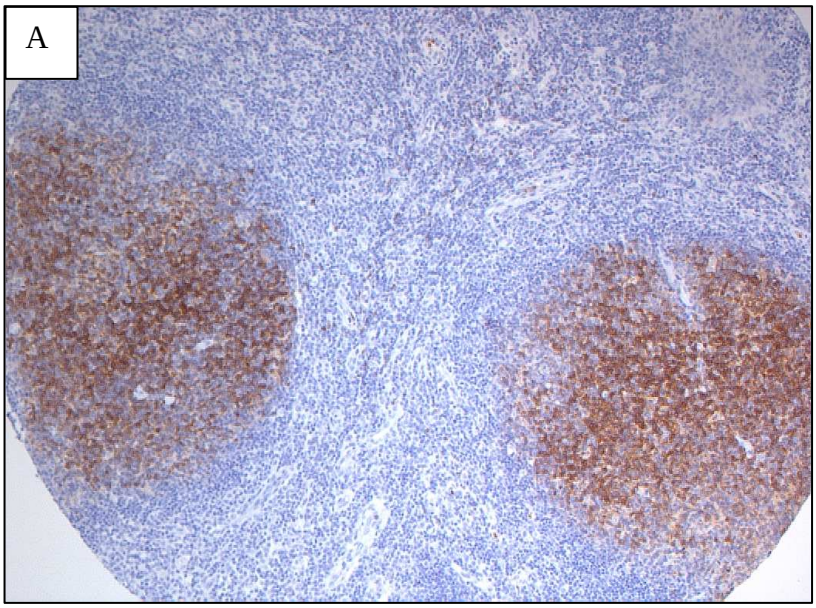
and prominent nucleoli, which are peripherally located. Follicular lymphoma is graded according to the number of centroblasts present. The World Health Organisation (WHO) recommends a three grade system which is based on the number of centroblasts in ten representative neoplastic follicles expressed per 40x microscopic field (hpf). Grade 1 contains 0-5 centroblasts/hpf, Grade 2 contains 6-15 centroblasts/hpf and Grade 3 cases more than 15 centroblasts/hpf (Metter GE et al. 1985, Nathwani BN et al. 1986). Grade 3 is further divided according to the number of centrocytes present; grade 3a contains more than 15 centroblasts/hpf with some remaining centrocytes. Grade 3b is composed of sheets of centroblasts with no centrocytes. Differences between grade 3a and 3b are not restricted to cytomorphologic features but are also seen at the immunohistochemical and cytogenetic level (Ott G et al. 2002). Grading appears to correlate with prognosis, grade 1 and 2 disease seem to have a similar indolent course with better overall survival but worse failure free survival (Martin AR et al. 1995) where grade 3 FL behaves in a more aggressive manner with a similar overall outcome to diffuse large B cell lymphoma (Bartlett NL et al. 1994; Wendum D et al. 1997). There is however, much variability in grading as this is a subjective technique (Metter GE et al. 1985; Anon 1997) which may impact treatment decisions.

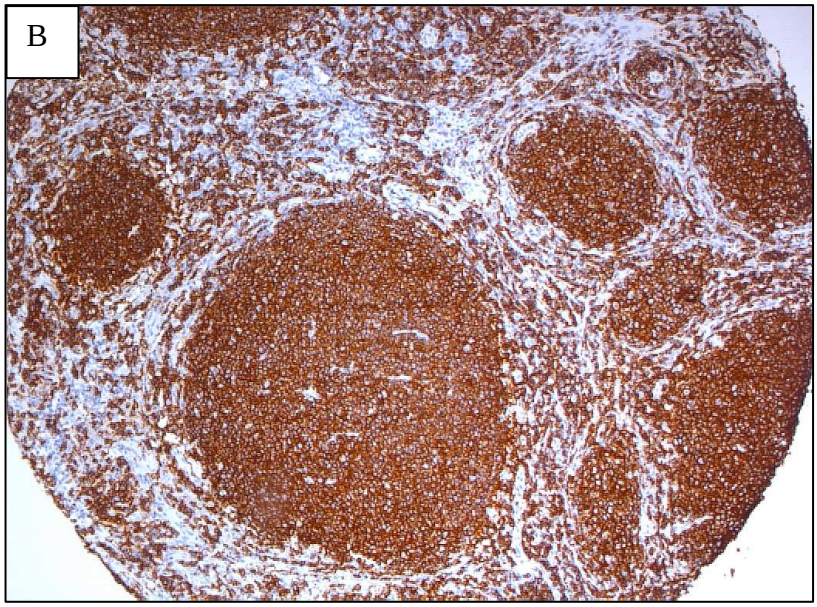
The WHO classification advocates that the pattern of nodularity is reported, expressed as percentage of nodular areas versus percentage of diffuse areas. The pattern is reported as follicular if >75% of the tumour is composed of nodules, follicular and diffuse if between 25-75% of the tumour is nodular, and focally follicular if <25% of the tumour is follicular. Patients with only focally follicular areas (<25%) have significantly worse freedom from progression and overall survival at 5 years in comparison to patients with >50% follicularity (Hu E et al. 1985). The median survival of patients with a follicular pattern was 68.2 months compared with 39.6 months in patients with follicular and diffuse pattern (Ezdinli EZ et al. 1987). The presence of 50% of a diffuse component predicts a worse overall survival and event free survival in grade 3 follicular lymphoma patients treated with an anthracycline based regime (Hans CP et al. 2003).

1.7.3 Immunocytochemistry

Immunophenotypically FL tumour cells are positive for pan B cell markers (CD20, CD19, CD22), CD10, Bcl-2 and Bcl-6 and negative for CD5. Bcl-2 is positive in the neoplastic follicles, which is the opposite pattern to that seen in reactive lymph nodes. CD23 and CD21 demonstrate the meshworks of the follicular dendritic cells within follicles (**Figure 14**). Assessment of the proliferation index using immunocytochemistry for Ki-67 has shown the proliferation index to be independently predictive of outcome in follicular lymphoma (Koster A et al. 2007).

Figure 14 Immunocytochemistry to demonstrate the immunophenotype of follicular lymphoma, A) CD10 positive in neoplastic follicles original magnification x10, B) CD20 positive in both follicles and interfollicular areas original magnification x10, C) CD21 positive meshwork in neoplastic follicles original magnification x10, D) CD23 positive meshwork in neoplastic follicles original magnification x10, E) BCL-2 positive in cells within the neoplastic follicle as well as interfollicular cells original magnification x10, F) BCL-6 positive in cell in neoplastic follicles original magnification x10, G) Ki-67 demonstrating occasional positive cells within neoplastic follicles original magnification x10

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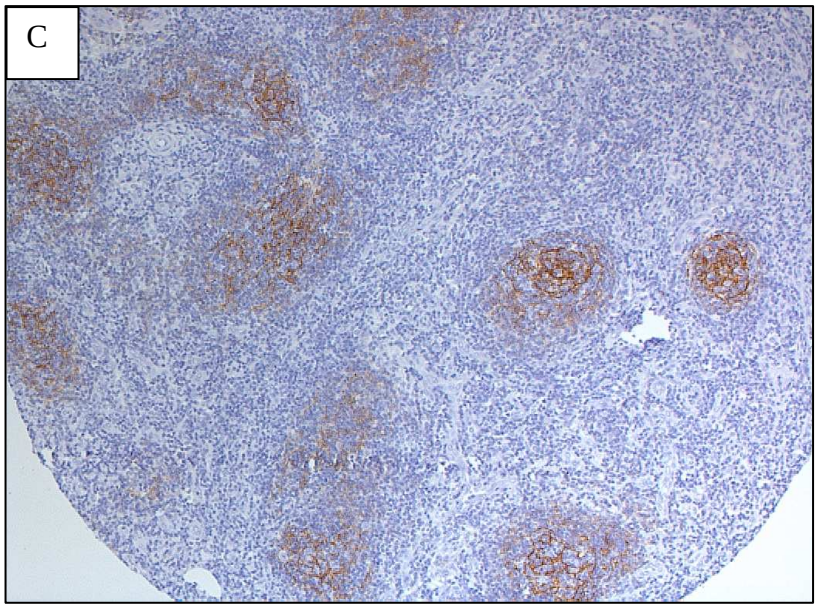
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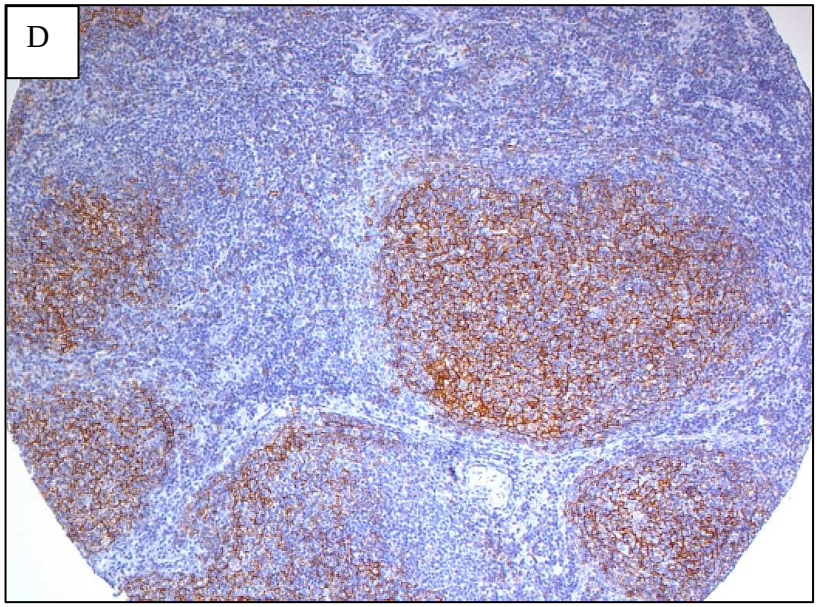
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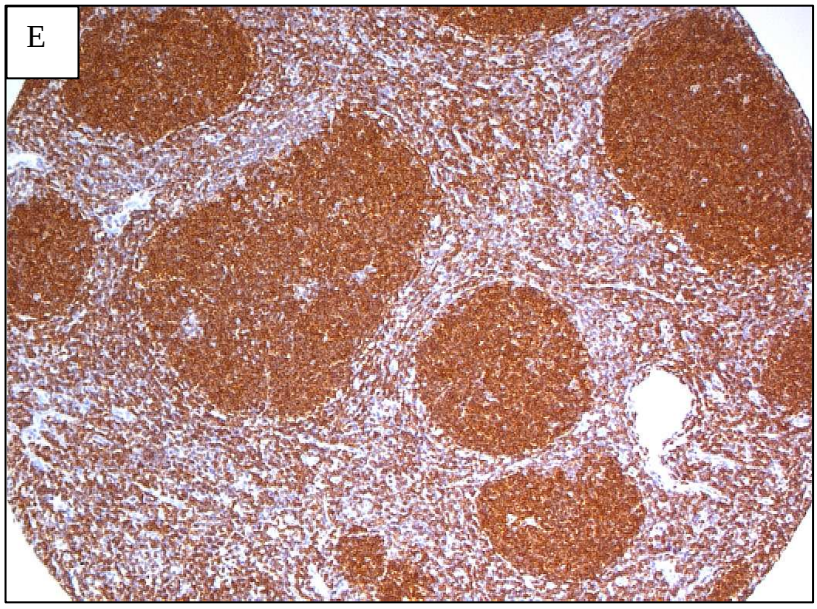
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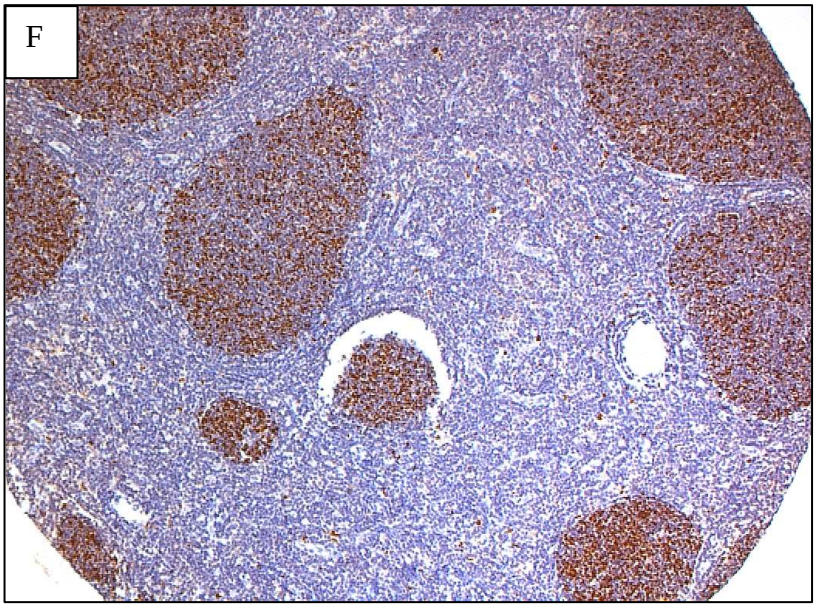
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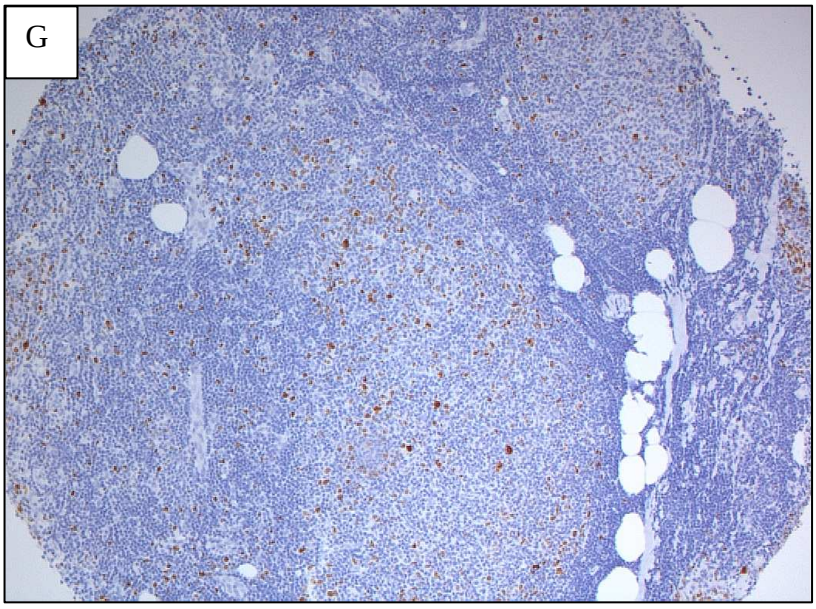
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1.7.4 Cytogenetic abnormalities

Most cases of follicular lymphoma show cytogenetic abnormalities (Tilly H et al. 1994). The most common genetic abnormality is a translocation involving chromosomes 14 and 18 (t(14;18)(q32;q21)). This is seen in approximately 70-95% of cases (Horsman DE et al. 1995) and results in the *BCL2* gene being juxtaposed to the immunoglobulin heavy chain (*IGH*) gene. This results in overexpression of the *BCL2* gene. Studies have shown that overexpression of Bcl2 in B cells grown *in vitro* prevents apoptosis even when growth factors are withdrawn (Nunez G et al. 1990). This genetic abnormality alone is not sufficient for the development of FL as *IGH/BCL2* gene rearrangements have been observed at low levels in healthy blood donors (Limpens J et al. 1995; Dolken G et al. 1996; Summers K et al. 2001) and in benign lymphoid tissue (Limpens J et al. 1991). Cytogenetic differences are seen between Grade 3a and Grade 3b cases, and a comparative study of FL grade 3a and 3b demonstrated t(14;18) in 73% of FL grade 3a cases but only in 13% of Grade 3b cases (Ott G et al. 2002). In the majority of cases of FL there are numerous other abnormalities most commonly involving chromosomes 1, 2, 4, 5, 13 and 17 or additions of X, 7, 12, or 18 (Tilly H et al. 1994). Abnormalities of 6q23-36 are detected in between 10-40% of B cell lymphomas. There may be three separate tumour suppressor genes within this region as deletions have been demonstrated at 6q21, 6q23 and 6q25-27 (Offit K et al. 1993). Breaks in 3q27 are observed in 44% of FL grade 3b in comparison to 18% of grade 3a (Ott G et al. 2002). The average number of genetic aberrations increases with grade with increased numbers of complex abnormalities in patients with higher grade tumours (Viardot A et al. 2002).

1.7.5 Treatment and Prognosis

Follicular lymphoma is a heterogeneous disease with varying survival rates and numerous treatment options. In some patients the disease is relatively indolent, however the lymphoma is usually not curable and the median survival is between 8 and 10 years after diagnosis (Gallager CJ et al.1986) (**Figure 12**). There are a proportion of patients with a significantly shorter duration of survival. Treatment options range from a conservative watch and wait policy for those patients who are symptom free with no apparent detrimental effect (Brice P et al. 1997, Advani R et al. 2004) through more aggressive treatments dependent upon the requirements of individual patients. Traditionally, the treatment options include radiotherapy, oral

chemotherapy and varying combinations of chemotherapeutic drugs (Cohen Y, Solal-Celigny P, Polliack A 2003). Radiotherapy has been shown to be curative for some patients with early stage low-grade FL (Mac Manus MP, Hoppe RT 1996). The alkylating agents cyclophophamide and chlorambucil have used either singly or in combination with other agents for many years. The use of single agent treatment has shown a response rate of between 50% and 75% in FL (Portlock CS et al. 1976; Gallagher CJ et al. 1986). The combination of alkylating agents with anthracyclines does not improve the response rate (Dana BW et al. 1993; Peterson BA et al. 2003) but may incur less risk of transformation (Young RC et al. 1988). The relatively new purine analogs have also shown efficacy in the treatment of non-Hodgkin's lymphomas (Redman JR et al. 1992; Hochster HS et al. 1992). Monotherapy with fludarabine produced response rates of 65% to 84% with complete remission in 37% to 47% of patients with previously untreated FL (Zinzani PL et al. 2000). In many cases, due to the protracted nature of the disease, various combinations of different treatment modalities are used. The combination of the purine analogs fludarabine and mitoxantrone with dexamethasone is effective in inducing complete remission in 47% of patients with recurrent and relapsed follicular lymphoma and a partial remission in 47% of patients. The median failure free survival time for patients in complete remission was 21 months (McLaughlin P et al. 1996). The development of a monoclonal antibody to the CD20 antigen has proved to be an exciting and extremely valuable addition to the range of therapeutic agents used to treat follicular lymphoma. Initial studies showed that the response rate to the drug was 48% with only mild toxicity. This figure is compatible to that seen in single agent chemotherapy (McLaughlin P et al. 1998). In addition, monoclonal antibodies can be used to deliver other therapeutic agents e.g. radioisotopes to the tumour cells (Witzig TE et al. 1999). A recent comparison of CHOP +/- rituximab versus fludarabine plus mitoxantrone (FM) +/- rituximab as initial treatment has shown that the FM combination is more effective than CHOP achieving complete remission in 68% of patients compared to 42%. No difference was found in terms of overall survival or progression free survival (Zinzani PL et al. 2004). Patients with disseminated disease who have not responded to conventional treatment or who have relapsed may benefit from intensive therapies such as high dose therapy (HDT) followed by autologous or allogeneic bone marrow transplantation (Apostolidis J et al. 2000; Bastion Y et al. 1995; Bierman PJ et al. 1997). The results from the studies on HDT followed by

stem cell transplantation indicate that prolonged failure free survival can be achieved by this option however prolonged survival or cure is not attained (Apostolidis J et al. 2000; Bastion Y et al. 1995; Bierman PJ et al. 1997).

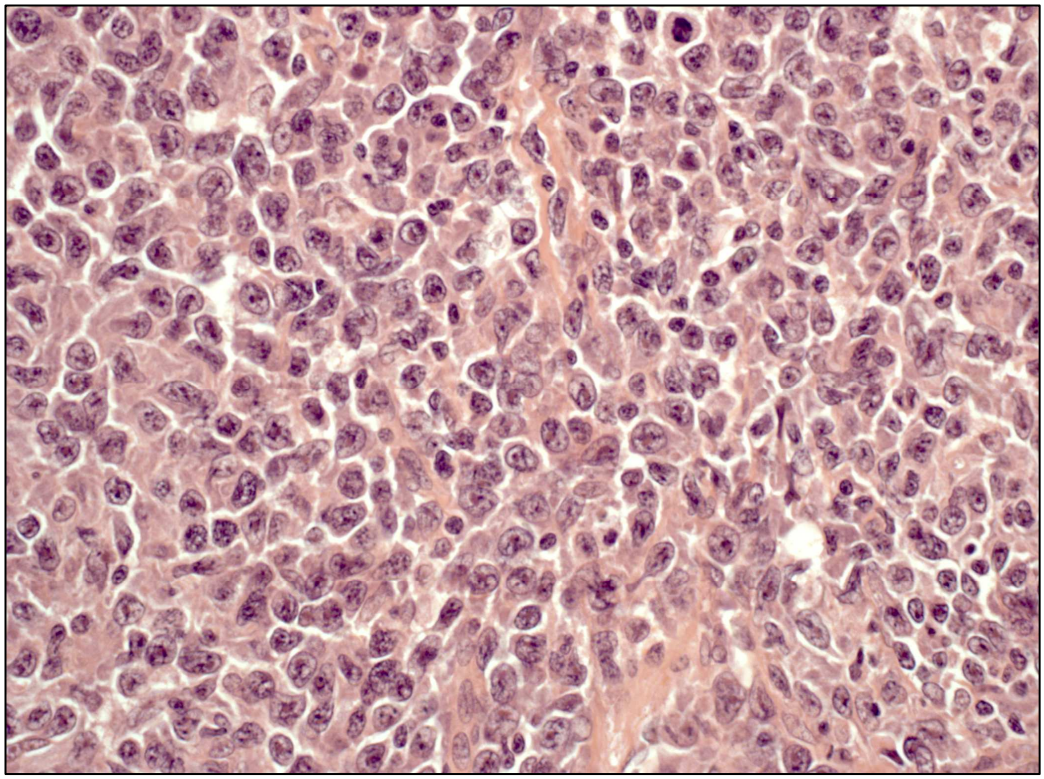
Unfortunately, there is no general consensus on which of these treatments should be used for FL (Solal-Celigny P et al. 2004).There is no clear cut pathway for treatment of patients with FL and although there is evidence of the use of treatment entities in FL there is no evidence as to the order in which these treatments should be used (Gribben JG 2007). The use of chemotherapy (single agent or combination) as initial treatment in comparison to watch and wait does not improve survival (Brice P et al. 1997; Young RC et al. 1988). It is also difficult to predict which patients have disease which will behave in an aggressive manner and in whom more proactive treatment is warranted. A recent multicentre analysis has identified five adverse prognostic factors which can segregate patients into three risk groups. The five risk factors selected are age (over 60 years compared to 60 years and under), Ann Arbor stage (III-IV vs I-II), haemoglobin level (less than 120g/L compared to 120g/L or over), number of nodal areas (more than 4 or 4 and under) and serum lactate dehydrogenase (LDH) level (high compared to normal or below). The three risk groups are low risk (0-1 adverse factor), intermediate risk (2 factors) and poor risk (3 or more adverse factors). The 5 year overall survival in the low risk group was 88.1%, the intermediate risk group between 57.4 and 70.9% and the poor risk group 43.6%. This index, named the Follicular Lymphoma International Prognostic Index (FLIPI), provides a consistent platform for comparison of clinical trials and evaluation of new treatments (Solal-Celigny P et al. 2004). In FL patients at first relapse/progression, the FLIPI, along with the presence of B symptoms, can predict five-year survival from progression and so may help in treatment decisions (Montoto S et al. 2004). There are difficulties with this index as stratification of patients into risk groups is limited. In addition, the index is rarely used as a tool to drive treatment choices in a clinical setting.

Typically the clinical course of follicular lymphoma is that of a relapsing and remitting disease with a reduced response to treatment and duration of remission over time with eventual death from disease (Johnson P et al. 1995).

1.8 Diffuse large B cell lymphoma (DLBCL)

Between 25 and 35% of patients with follicular lymphoma transform to diffuse large B cell lymphoma (DLBCL) (Acker B et al. 1983; Gallagher CJ et al. 1986; Montoto S et al. 2007) (**Figure 15**). This is a neoplasm of large B cells which typically replaces tissue in a diffuse pattern. Within the disease entity several morphological variants have been described by the WHO classification system. This disease can also arise in patients without a pre-existing lymphoid neoplasm, so called *de novo* DLBCL.

Figure 15 Morphology of DLBCL stained with haematoxylin and eosin original magnification x40



1.8.1 Clinical Features

Patients with DLBCL typically present with single disease sites however the disease is aggressive and will result in death if not treated. A proportion of patients present with extranodal disease particularly in the skin and gastrointestinal tract.

1.8.2 Immunocytochemistry

In keeping with the B cell origin the tumour cells are positive for pan B cell markers (CD19, CD20 and CD22). There is variable expression of CD5 (approximately 10% of cases) and CD10 (approximately 25% of cases). Expression of Bcl-2 is more commonly seen and is observed in between 30 and 50% of cases. The proliferation fraction, as expressed by ki67 expression, is high. Typically there is loss of the FDC meshworks identified by CD21 and CD23 immunocytochemistry.

1.8.3 Cytogenetic abnormalities

The most common translocation in DLBCL is t(14;18) which is detected in 20 - 30% of cases (Weiss LM et al. 1987; Offit K et al. 1991a). Translocation of the c-*MYC* gene to the immunoglobulin heavy chain gene; t(8;14) is also observed in a proportion of cases (Ladyani M et al. 1991) and confers a worse outcome with aggressive disease (Vitolo U et al. 1998). Additional abnormalities have been observed which correlate with clinical outcome; patients with breaks at 1q21-23 or 1p32-36 had a shorter remission time and patients with breaks at 6q21-25 were less likely to achieve remission (Offit K et al. 1991).

1.9 Transformation of FL to DLBCL

Transformation of FL to aggressive morphology, usually DLBCL, is an area fraught with difficulty. The mechanisms of transformation are unclear and as a result, prediction of patients who will transform is difficult. Indeed the incidence of patients who transform varies widely. Clear evidence of transformation is only provided by histological analysis of a biopsy however the biopsy rate is extremely variable. In some centres, the presence of clinical symptoms, rapid enlargement of lymph nodes or elevation of biochemical markers are sufficient for the diagnosis to be made and treatment instigated. Numerous studies over the years have produced data on this phenomenon and the incidence ranges from 12% to 68%. A study in 1983 observed a 12% incidence of transformation in 83 patients with untreated FL followed for a

median of 50 months (Horning S; Rosenberg SA 1984). Post-mortem investigations have demonstrated a much higher incidence of transformation; 68% (Garvin et al. 1983). Data from St Bartholomew's hospital, based on patients with histological evidence of transformation, demonstrated transformation in 88 patients out of a possible 328 prior to 2000. Transformation occurred during the time between initial diagnosis of FL and 16 years after diagnosis however no patient transformed after 16.2 years (Montoto S et al. 2007).

A study by Bastion et al. identified three factors which indicated patients likely to transform; failure to achieve complete remission after primary treatment, low serum albumin (<35 g/L) and raised β_2-microglobulin (>3 mg/L) at diagnosis. In their cohort of patients the median survival time after transformation was only seven months and accounted for 44% of deaths (Bastion Y et al. 1997), in keeping with the clinical observation that patients who undergo transformation have a poor prognosis. This observation is again demonstrated in the data produced by St Bartholomew's Hospital (**Figure 16, Figure 17**).

Figure 16 A Kaplan-Meier curve to show survival from transformation (n=88) in patients at St Bartholomew's Hospital (Montoto S et al. 2007)

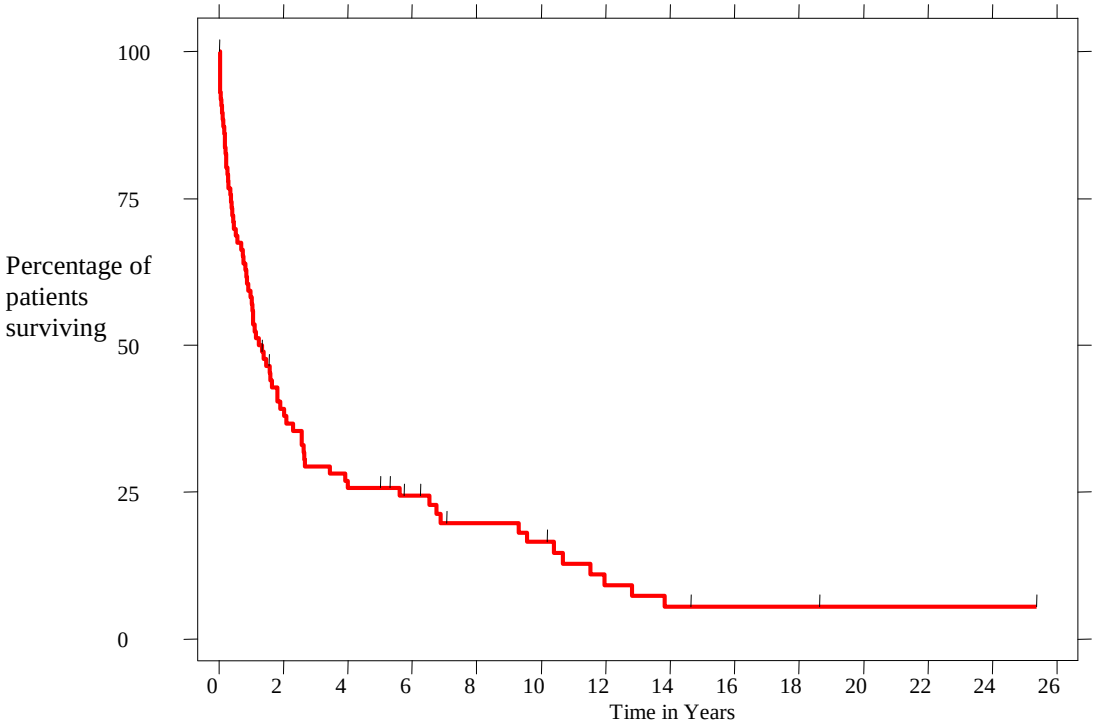
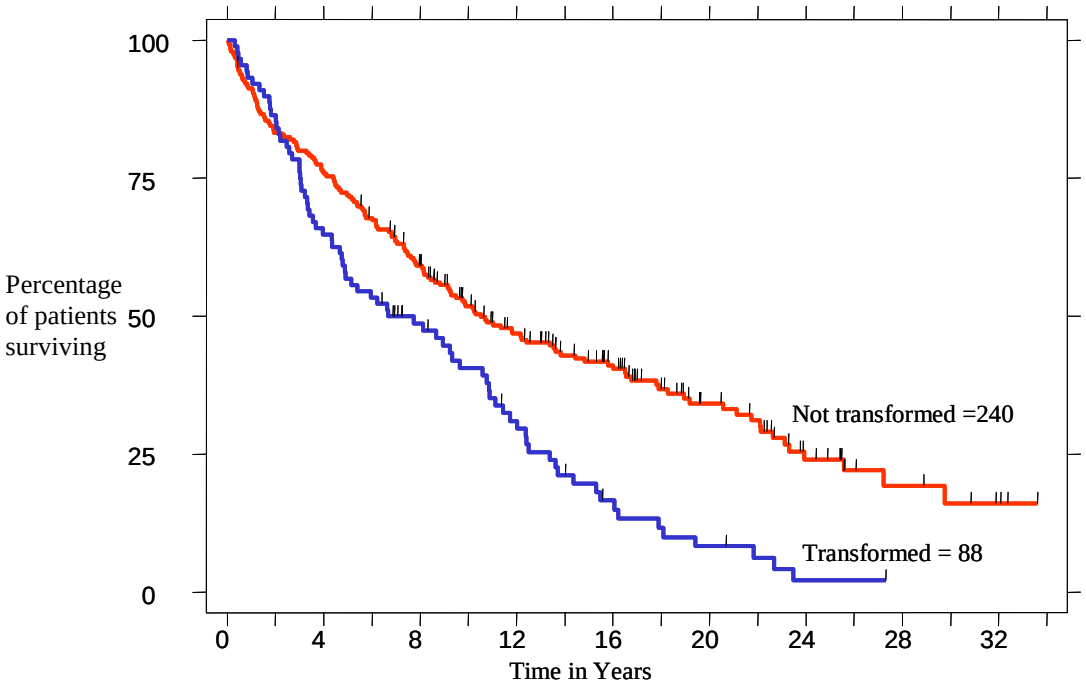


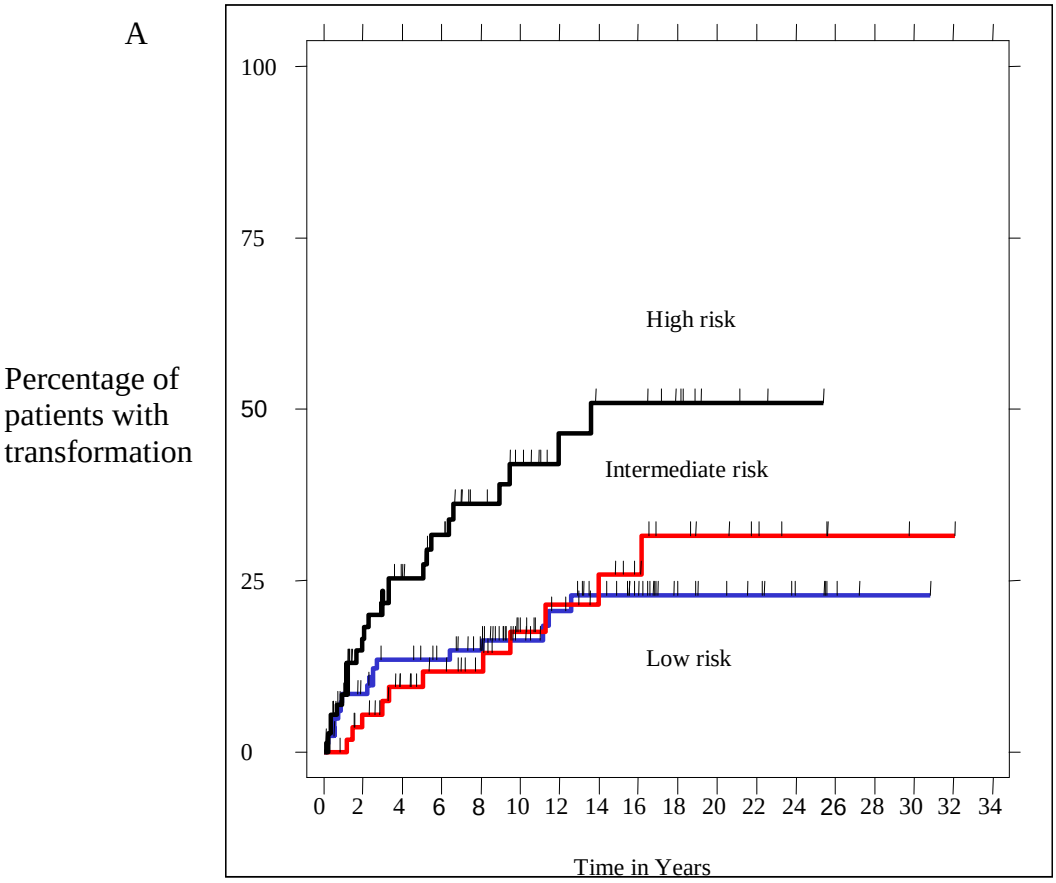
Figure 17 Overall survival according to transformation (Montoto S et al. 2005; 2007)



A large study of 276 patients assessed the main biological and clinical variables with respect to correlation with transformation and survival. Of the 276 patients 30 (11%) had undergone histological transformation after a median follow up of 6.5 years. The risk of transformation at 10 years and 15 years was 15% and 22% respectively. Grade 3 histology and the FLIPI score were of prognostic significance, however in grade 1-2 patients only the FLIPI predicted transformation (Gine E et al. 2006). The study at St Bartholomew's hospital also showed the risk of transformation was higher in patients with advanced stage at presentation and a high risk FLIPI (**Figure 18**) (Montoto S et al. 2005; 2007).

Figure 18 Prognostic factors for transformation at diagnosis in patients from St Bartholomew's Hospital (Montoto S et al. 2005; 2007) A) FLIPI at presentation B) Stage at presentation

A



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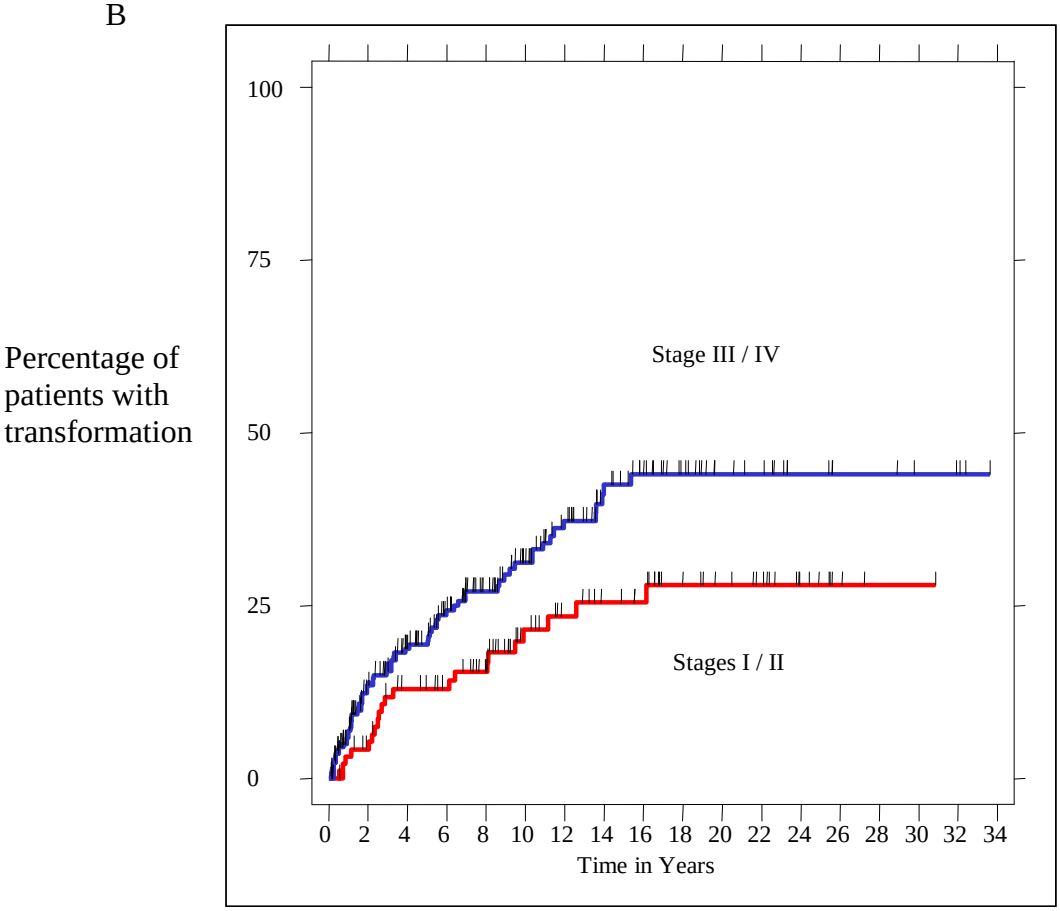
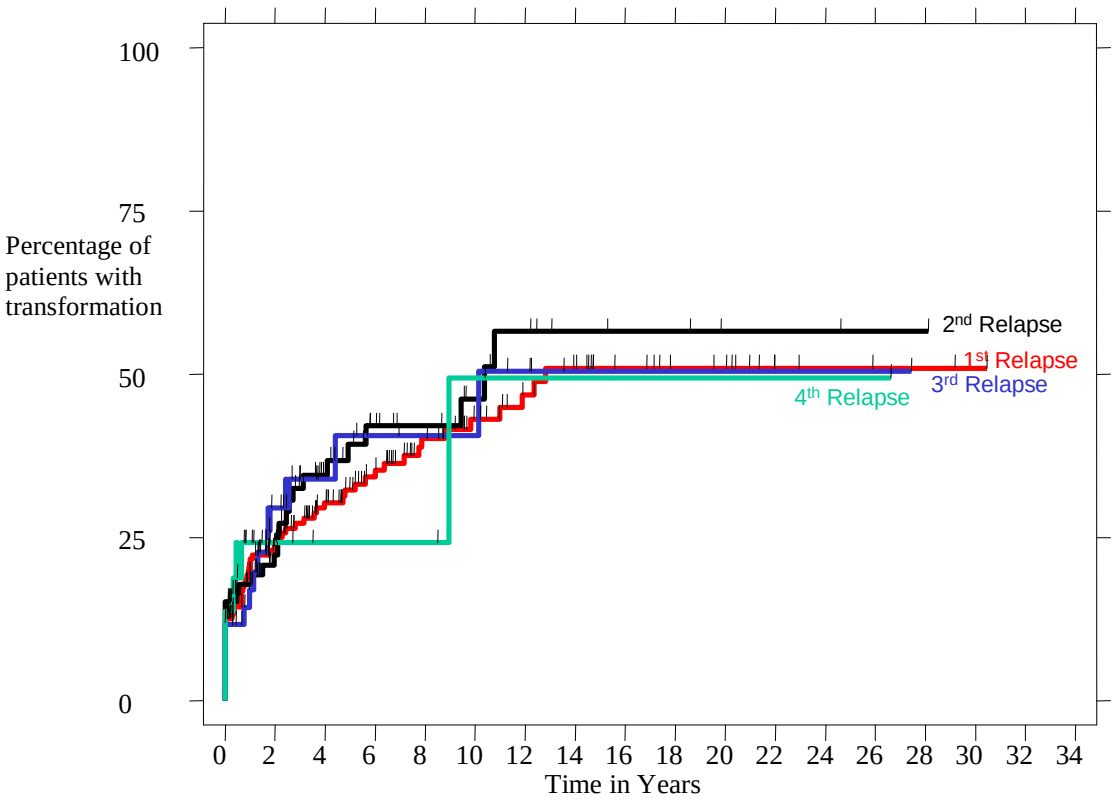


The natural history of FL is an indolent disease typically characterized by numerous relapses and remissions. The relationship of relapse and disease progression and transformation is also unclear. Analysis of the data from St Bartholomew's Hospital shows a constant rate of transformation for each episode of relapse; 17%, 14%, 14% and 14% for 1st, 2nd, 3rd and 4th episodes respectively (**Figure 19**) (Montoto S et al. 2005; 2007).

Figure 19 Risk of transformation at each disease relapse in patients from St Bartholomew's Hospital (Montoto S et al. 2005; 2007)



1.9.1 Mechanisms of transformation

As indicated previously, the mechanisms by which transformation of FL to DLBCL occur are incompletely understood. A number of cytogenetic abnormalities, both gains and losses, have been reported which may be important in the pathogenesis of transformation.

1.9.1.1 Chromosome gains

Gains in the region 2p13-16 were detected by comparative genomic hybridization (CGH) and PCR analysis of this region identified amplification of the *REL* gene in half of a small group of patients with FL who subsequent transformed (Goff LK et al. 2000).

Gain at chromosome 7 has been observed in 30% of Non-Hodgkins lymphoma cases with the t(14;18) and was associated with a diffuse pattern. Gain of chromosome was present in 52% of patients with a diffuse pattern compared to 15% of patients with a follicular growth pattern (Armitage JO et al. 1988). A larger study identified a gain of chromosome 7 as the most common cytogenetic abnormality in t(14;18) lymphomas (Johansson B, Mertens F, Mitelman F 1995). A further study identified a gain of chromosome 7, in particular 7p, in 5 out of 6 cases at the time of transformation. Loss of 9q in combination with gain of 7p was observed in 4 out of 5 cases of FL grades 1 or 2 which transformed to DLBCL (Boonstra R et al. 2003).

Gains in chromosome 12q12-14 were detected in 52% of transformed cases but were not present in the preceding FL sample. Patients with gains in this region tended towards a poorer outcome in comparison to patients without this cytogenetic alteration although this did not reach statistical significance (Hough RE et al 2001). Analysis of three genes which map to this area; *CDK2, CDK4* and *GADD153* in a set of 44 paired FL/DLBCL samples (22 patients) demonstrated deregulation of *CDK2* and *CDK4* genes at both a genetic and protein level suggesting a functional role for these genes in the transformation process. The expression of GADD153 protein was not detected in most cases (Al-Assar O et al 2006). Patients with abnormalities of 6q23-26 were at significantly higher risk of transformation to DLBCL (Tilly H et al. 1994).

1.9.1.2 Chromosome losses

A commonly implicated gene in transformation is *TP*53 (Sander CA et al. 1993) which is located at chromosome 17p13. Loss of 17p has been linked to both inferior survival and a shorter time to transformation (Tilly H et al. 1994). The possible role of *TP53* in transformation will be discussed in Chapter 2.

The presence at time of diagnosis of FL of a deletion involving bands 6q23-26 is significantly associated with increased risk of transformation and a shorter survival time (Tilly H et al. 1994).

Losses of 5p and 8q have also been observed in transformation samples in comparison to antecedent FL biopsies (Hough RE et al 2001).

1.9.1.3 $p16^{INK4a}$

The use of polymorphic microsatellite markers in the 9p21 region, which contains the tumour suppressor genes $p15^{INK4B}$ (CDKN2B) and $p16^{INK4A}$ (CDKN2), in a series of 11 paired FL and transformed DLBCL samples, identified deletions exclusively in the DLBCL samples in 8 of 11 cases (73%). Six of these were homozygous (54%) and two were hemizygous (18%). Immunocytochemistry demonstrated positive staining for p16 in FL tumour cells in nine available cases which were evaluable, with reduced protein expression in 4 of the 9 (44%) cases of DLBCL. All 4 cases demonstrated homozygous mutation deletions at 9p21. The remaining 5 cases with p16 protein expression retained at least one 9p21 allele (Elenitoba-Johnson KSJ et al. 1998). A further study identified *p16* alterations as an infrequent event in NHL but loss of protein expression was associated with aggressive tumours and a potential role in transformation (Pinyol M et al. 1998). These results suggest genetic loss in the region of 9p21 involving *p16* and possibly *p15* may be important in the process of transformation.

1.9.1.4 *BCL-2*

Alterations in *Bcl-2* are implied as a result of the t(14;18) in FL however, additional genetic abnormalities have been identified upon transformation to DLBCL. Biopsies from eight patients with FL were analysed, of which 6 transformed to DLBCL. A high level of conservation of the *bcl-2* gene was observed during the t(14;18) and

morphological alteration. Three DLBCL samples contained 11 point mutations in the open reading frame which were not present in the antecedent FL samples suggesting that transformation may be associated with somatic point mutation of the *bcl-2* gene (Matolcsy A et al. 1996). The presence of *bcl-2* rearrangement in *de novo* DLBCL correlates with worse outcome (Yunis JJ et al. 1989).

1.9.1.5 *BCL-6*

An important gene in the pathogenesis of *de novo* DLBCL is the *Bcl-6* proto-oncogene which is located on 3q27. The product is a 79-kilodalton protein homologous with zinc finger-transcription factors (Ye BH et al. 1993a, Ye BH et al. 1993b) and is essential for germinal centre formation (Ye BH et al. 1997). Rearrangements of *Bcl-6* have been detected in approximately 40% of cases of DLBCL (Lo Coco F et al. 1994) and correlate with a favourable clinical outcome (Offit K et al. 1994). In a third of DLBCL samples the *Bcl-6* gene is truncated within the 5' noncoding sequences indicating that its expression is deregulated (Ye BH et al. 1993b). *Bcl-6* is able to function as a transcriptional repressor (Seyfert VL et al. 1996) and is expressed by centroblasts and centrocytes in germinal centres as well as CD4 T lymphocytes. The presence of Bcl-6 protein in FL and DLBCL independent of gene rearrangement suggests that *Bcl-6* is regulated during B cell development and plays a role in germinal centre formation (Cattoretti G et al. 1995). Expression of the Bcl-6 protein is seen in both FL and DLBCL (Onizuka T et al. 1995). The frequency of alterations of the *Bcl-6* gene in FL is much lower than *de novo* DLBCL ranging from 6-14% (Bastard C et al. 1994; Lo Coco F et al. 1994; Otsuki T et al. 1995). The incidence of *Bcl-6* gene rearrangements in transformed FL varies; the study by Otsuki et al. 1995 demonstrated an incidence of 17.3%. Eleven cases of transformed FL with the antecedent FL biopsies were available and in only two of these cases was *Bcl-6* rearranged. In both cases the rearrangements were present in both FL and transformed samples suggesting *Bcl-6* rearrangements are not temporally associated with transformation (Otsuki T et al. 1995). In contrast, a study by Lossos and Levy observed new mutations in *Bcl-6* in 5 out of 7 transformed FL samples which were not present in the preceding FL biopsy. The numbers of mutations ranged from 1 to 6 and clustered in areas of the 5' noncoding regulatory region of the *Bcl-6* gene (Lossos IS, Levy R 2000). This result suggests that deregulation of *Bcl-6* may be important in the pathogenesis of transformation. Bcl-6

has been demonstrated to repress the gene coding for ATR which is important in sensing DNA damage. Reduction in the levels of ATR in B lymphocytes by Bcl-6 allowed primary centroblasts to survive DNA damage. This was mediated by reduced phosphorylation of histone H2AX and the checkpoint regulator Chk1. The levels and function of ATR were restored by CD40-mediated disruption of the Bcl-6 complex (Ranuncolo SM et al. 2007). In addition *Bcl-6* can downregulate the expression of *p53* (Phan RT, Dalla-Favera R 2004).

Much of the emphasis has been on evaluation of mutations and their contribution to the process of transformation. There has been very little investigation into the role which the microenvironment has to play in transformation and a single event which results in transformation has not been identified. This suggests that there may be several pathways which result in transformation of FL to DLBCL and an interaction between mutations and the microenvironment may play a role in this process. Transformation is such an adverse event that the ability to predict those patients likely to transform at diagnosis would be extremely valuable.

1.10 The role of the microenvironment in lymphomagenesis

Speculation on the role that the immune system plays in the development and progression of malignant tumours is not a recent development. Virchow suggested in 1863 that there may be a causal relationship between chronic inflammation and the development of cancer (Balkwill F, Mantovi A 2001). There are several pieces of evidence which support this idea. In those malignancies where infectious agents are a known causal agent the initial response to the infection is inflammatory, for example Helicobacter pylori infection increases the risk of developing gastric cancer by an odds ratio of 2.77 (Forman D et al. 2001). Infection with Hepatitis C virus is a well recognized risk factor for the development of hepatocellular carcinoma (Colombo M et al. 1989; Hasan F et al. 1990). In addition, the increased risk of patients with autoimmune disease, such as ulcerative colitis, in developing malignancy indicates a role for inflammation in oncogenesis (Gyde SN et al. 1988). These findings are counterintuitive to the known role of the immune system. There is an expectation that the immune system should mount an anti-tumour reponse however the tumour – immune microenvironment interaction appears to be extremely complex and this is reflected by the numerous and often conflicting observations about the numbers and types of tumour infiltrating lymphocytes (TILs) within malignancies and correlation of these with prognosis for example, in patients with melanoma there have been reports of improved prognosis in patients with TILs (Clemente CG et al. 1996) whilst other reports have found no association (Barnhill RL et al. 1996). Recent data suggests that the absence of TILs in a primary cutaneous melanoma predicts sentinel lymph node metastasis in patients, SLN involvement is the most important predictor of survival in patients with melanoma (Taylor RC et al. 2007).

1.10.1 TILs in FL

There are several lines of evidence that the immune microenvironment is important in the biology of FL. Numerous CD4 T lymphocytes have been observed predominantly in the interfollicular areas, as well as the neoplastic follicles of FL (Swerdlow SH et al. 1985). Significantly more CD4+ T lymphocytes have been observed in the biopsies of untreated FL patients who underwent spontaneous regression than in the biopsies of those who did not (Strickler JG et al. 1988). These observations suggested that T lymphocytes might have an important role in FL. Gene expression profiling of FL biopsies from untreated patients produced two gene

expression signatures which allowed grouping of patients into quartiles with large differences in survival. These signatures were demonstrated to be a result of gene expression by tumour infiltrating cells and were independent of clinical parameters. The two signatures were denominated immune-response 1 (IR1) and immune-response 2 (IR2). The IR1 response was associated with favourable prognosis and included genes coding for T lymphocyte markers and macrophages markers. The IR2 response associated with poor prognosis, and contained genes for markers expressed by macrophages, dendritic cells or both (Dave SS et al 2004). Supporting evidence at a protein level was provided by immunohistochemical analysis of 99 diagnostic FL lymph node biopsies which demonstrated a correlation between increased numbers of CD68 positive macrophages and poor prognosis. However, analysis of T lymphocyte markers did not identify an improved prognosis with their expression as suggested by the IR1 response (Farinha P et al. 2005).

1.10.2 Regulatory T lymphocytes

There is growing interest in the role of the T-regulatory (Treg) cell subset in human diseases. The demonstration of T lymphocytes which could suppress the immune system was shown by transfer of antigen experienced T lymphocytes into naïve mice producing antigen specific tolerance (Gershon RK, Kondo K 1971). It was not until the identification of a population of CD4 T lymphocytes with high levels of CD25 expression which could prevent autoimmunity in mice, that interest in these cells became widespread (Sakaguchi S et al. 1995). These CD4+CD25+ regulatory T cells account for approximately 10% of CD4+ T lymphocytes in the spleen and lymph nodes of normal mice (Cederbom L et al. 1998) but only 1-2% of the CD4 postive T lymphocytes in humans (Baecher-Allan C et al. 2001) In murine models CD25 is an acceptable marker to delineate a Treg as the animals are maintained in pathogen free environments. In humans, exposure to antigens produces an increase in CD25 positive effector T lymphocytes. Therefore a marker specific for T regulatory cells was required. Genetic mutations of the transcription factor, *FOXP3,* in both mice and humans were identified as the defect resulting in fatal autoimmune disease in the *scurfy* mice (Brunkow ME et al. 2001) and human X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy (IPEX) (Bennett CI et al. 2001) Using a *Foxp3*^{gfp} reporter allele in mice, Foxp3 expression was demonstrated to be restricted to only Tregs (Fontenot JC et al. 2005a). Deletion of germline *Foxp3* in mice

resulted in a lack of Tregs in the thymus and development of autoimmune disease which could be ameliorated by the adoptive transfer of Tregs into Foxp3 deficient neonates (Fontenot JD et al. 2003; Fontenot JD et al. 2005a). Furthermore, the continued expression of Foxp3 is required in mature Tregs to maintain the suppressive function of Tregs. Loss of *Foxp3* in these cells allowed reversal of the Treg transcriptional program and the production of proinflammatory cytokines with pathogenic potential. (Williams LH, Rudensky AY 2007). Regulatory T cells originate in the thymus (Modigliani Y et al. 1996).

1.10.2.1 Types of regulatory T cells

Several types of T regulatory cells have been defined. Naturally occurring Tregs, which are produced in the thymus and are exported as fully functional cells (Itoh M et al. 1999). The other subtypes are induced regulatory cells; Tr1 cells are generated from chronically activated CD4+ T lymphocytes in both mice and humans in the presence of IL10. This subset of cells produce high levels of IL-10, low levels of IL-2 and no IL-4 and can suppress the proliferation of CD4+ T lymphocytes in response to antigen. They can also prevent colitis induced in SCID mice by pathogenic CD4+CD45RB^{high} splenic T lymphocytes (Groux H et al. 1997). Th3 cells, can be generated in vivo after oral administration of antigen and produce TGF-β as well as IL-4 and IL-10 (Chen Y et al. 1994). Finally a small population of regulatory cell which express CD8 as opposed to CD4 have been identified (Xystrakis E et al. 2004).

1.10.2.2 Functions of regulatory T cells

Functionally Tregs do not proliferate when stimulated via the T cell receptor (TCR) but can proliferate when stimulated by interleukin-2 (IL-2) (Thornton AM, Shevach EM 1998; Thornton AM, Shevach EM 2000). Indeed IL-2 signalling is required for maintenance of optimal Treg function in vivo (Fontenot JD et al. 2005b). When activated by their TCR, Tregs suppress proliferation of CD4+ T lymphocytes (Thornton AM, Shevach EM 1998) and CD8+ T lymphocytes (Piccirillo CA, Shevach EM 2001) by inhibiting IL-2 mRNA transcription (Thornton AM, Shevach EM 1998). The suppression is dependent on cell-contact and is cytokine independent in those Tregs produced in the thymus (Thornton AM, Shevach EM 2000; Piccirillo CA, Shevach EM 2001). Tregs are also able to suppress production of IL-4 and IFN-

γ suggesting that Foxp3 may block transcriptional activators required for activation of multiple cytokine genes. In support of this theory, Foxp3 was demonstrated to associate with the nuclear factor of activated T cells (NFAT) and NF-κB in murine models. This prevented endogenous expression of the target genes of NFAT and NF-λB which include cytokine genes (Bettelli E, Dastrange M, Oukka M 2005). The target genes of Foxp3 are key modulators of T cell activation and function and the major effect of Foxp3 is to suppress the activation of genes responsible for T cell stimulation (Marson A et al. 2007). Although Foxp3 function is required for regulatory T cell suppressor function it also increases pre-established molecular features of regulatory T cells including anergy and dependence on IL-2. In addition, Foxp3 modifies cell surface and signalling molecules including suppressing the cyclic nucleotide phosphodiesterase 3B which is essential to regulatory T cell maintenance (Gavin MA et al. 2007).

1.10.2.3 The role of regulatory T cells in cancer

The role of the T-regulatory (Treg) cell subset in the tolerance or suppression of malignancy is now being unravelled. Infiltrating Tregs are associated with suppression of tumour specific T cell immunity and poor prognosis in ovarian carcinoma (Curiel, TJ et al. 2004) amongst numerous other epithelial malignancies (Loddenkemper C et al. 2006, Fu J et al. 2007). Increased numbers of regulatory T cells have been observed in the metastatic lymph nodes of patients with malignant melanoma which actually inhibit the function of infiltrating cytotoxic T lymphocytes and CD4 positive non regulatory T lymphocytes (Viguier M et al. 2004). In haematological malignancy the picture is much less clear. Functional Treg have been identified in B-Non Hodgkin's Lymphomas (Carrreras J et al. 2006; Yang ZZ et al. 2006) and expression of forkhead transcription factor 3, (FOXP3), the defining antigen of Tregs, has been shown to correlate with improved outcome in FL (Carreras J et al. 2006).

FOXP3 has three functional domains: a C2H2 zinc-finger motif (amino acids 200-223) whose function is unknown, a leucine-zipper-like motif (aa 240-261) and the carboxy-terminal forkhead domain (aa 338-421) which binds DNA. Mutations causing IPEX having been detected in the forkhead domain, the leucine zipper and the N-terminal portion of the protein (Lopes JE et al. 2006). Mutation in the leucine

zipper domains impairs dimerization and suppressive function (Chae WJ et al. 2006). Foxp3 is constitutively localized in the nucleus and this requires sequences at both the amino and C-terminal ends of the forkhead domain. A functional domain in the N-terminal half of Foxp3 is required for repression of transcription from a constutively active and a NF-AT inducible promoter (Lopes JE et al. 2006). FOXP3 protein is detected as a doublet (Yagi H et al. 2004; Walker MR et al. 2003, Scotto L et al. 2004) with the lower band of the doublet representing a splice isoform lacking exon 2 (Yagi H et al. 2004, Scotto L et al. 2004). The splice variant lacking exon 2 and a newly identified variant lacking both exons 2 and 7 are both functional inhibitors of human CD4+ T-cell activation (Smith EL et al. 2006).

1.10.3. Tumour associated macrophages

Tumour associated macrophages (TAMs) have been analysed in numerous tumours including breast (Leek RD et al. 1996), bladder (Hanada T et al. 2000), and prostate (Lissbrant IF et al. 2000) cancer and have been associated with inferior survival. In some malignancies, stomach (Ohno S et al. 2003), colorectal (Funada Y et al. 2003) and melanoma (Piras F et al. 2005) high numbers of TAMs have been correlated with favourable prognosis. In follicular lymphoma the lymphoma-associated macrophage (LAM) content is an independent predictor of survival with increased numbers being associated with poor overall survival (Farinha P et al. 2005).

1.10.4 The role of the immune microenvironment in transformation of FL to DLBCL

The role the microenvironment may play in the process of transformation of FL to DLBCL has not been fully explored. The observations made in FL and in *de novo* diffuse large B-cell lymphoma suggest that it may play an important role. The data in *de novo* diffuse large B-cell lymphoma is from several studies with conflicting results; low numbers of CD8 positive T lymphocytes were associated with a poor outcome in one study (Lippman SM et al. 1990) however the presence of >15% of activated cytolytic T lymphocytes was strongly associated with failure to reach complete remission, with a poor progression-free and overall survival in a different study (Muris JJ et al. 2004). The presence of high numbers of CD4 positive T lymphocytes was associated with significantly longer 5 year failure-free survival as well as being an independent predictor of relapse free and overall survival (Ansell SM et al. 2001). This was also demonstrated in a study using flow cytometry where

cases with high numbers of T lymphocytes (>20%) and a CD4:CD8 ratio of >2.0 demonstrated significantly improved overall survival (Xu Y et al. 2001). A recent study using immunocytochemistry on paraffin embedded tissue in 195 patients has described significantly improved outcome in patients with a small number of cytotoxic T-cell intracytoplasmic antigen-1 (TIA-1) however, there was no correlation with the number of regulatory T cells (Hasselblom S et al. 2007). There has been little published work in transformation although Carreras et al. (2006) demonstrated that increased number of FOXP3 positive cells were associated with improved survival in FL patients and a marked reduction in FOXP3 positive cell number was observed on transformation of FL to DLBCL (Carreras J et al. 2006).

1.11 Aims and Objectives

Follicular lymphoma is a heterogeneous disease typically characterized by numerous relapses and remissions. At presentation, it is extremely difficult to predict which patients will have aggressive disease and die rapidly of disease and those in whom the disease pursues a more indolent cause. In addition, a substantial proportion of patients will transform to aggressive histology which is a clinically catastrophic event. There has previously been a great deal of work on the genetic defects which contribute to the development of FL as well as transformation to DLBCL. Recently, there has been interest in the role the microenvironment plays in lymphomagenesis, disease progression and transformation of FL to DLBCL. The main hypothesis of this thesis is that the immune microenviroment has an impact on the natural history of follicular lymphoma. The primary aim of this thesis is to examine the role the microenvironment has in the natural history of follicular lymphoma and in patient outcome. In addition, a limited study of some putative molecular mechanisms, as well as the impact of the immune microenvironment in transformation of FL to DLBCL are investigated.

CHAPTER TWO:

Materials and Methods

2.1 Materials and method for cell culture

2.1.1 Materials

RPMI medium

500 milliliters (mLs) of RPMI 1640 medium with Glutamax I (Gibco) was enriched with 10% foetal calf serum (FCS) (v/v) (Gibco) and 1% streptomycin/penicillin added. The RPMI solution was then filtered through a 0.2 millimetre (mm) mesh of a Stericup vacuum driven filtration system (Millipore, Billerica, MA).

2.1.2 Methods

All glassware used for cell culture was heated to 220˚C for 12 hours to ensure sterility from endotoxins. Cells frozen in liquid nitrogen were removed and thawed rapidly at 37˚C and the sample added dropwise to 10 mLs of RPMI/FCS/antibiotic solution. The solution was centrifuged for 5 minutes 1200rpm at 19˚C and the supernatant removed. The cell pellet was resuspended and 10mLs of RPMI/FCS/antibiotic solution added. The solution was centrifuged again for 5 minutes 1200rpm at 19˚C and the supernatant removed. The cell pellet was resuspended with 10 mLs of RPMI/FCS/antibiotic solution. 10mLs of RPMI/FCS/antibiotic solution was placed in a sterile medium sized culture flask (200mLs) (Nunc, Denmark) and the cell suspension added. Cells were grown as a monolayer in sterile flasks in a humidified atmosphere at 37˚C containing 5% carbon dioxide. At 80% confluency the cells were passaged by trypsinisation. The media was discarded and 10mLs of 0.05% tryspin was added to the flask and incubated for 4 minutes at 37°C. The cells were detached from the flask and were removed. Cells were pelleted by centrifugation at 1,300g for 5 minutes, re-suspended in medium and split and transferred into new flasks.

2.2 Materials and methods for Flow cytometry

2.2.1 Materials

Buffer (Miltenyi Biotec Inc, Earhart Avenue, CA, USA)

Phopshate buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2mM EDTA

FcR Blocking Reagent (Miltenyi Biotec Inc, Earhart Avenue, CA, USA)

Human IgG

Fixation and Permeabilization solution (Miltenyi Biotec Inc, Earhart Avenue, CA, USA)
Fixation/Permeabilization Solution 1 (Miltenyi Biotec Inc, Earhart Avenue, CA, USA) diluted 1:4 with Fixation/Permeabilization Solution 2 (Miltenyi Biotec Inc, Earhart Avenue, CA, USA)

Permeabilization Buffer (Miltenyi Biotec Inc, Earhart Avenue, CA, USA)
Dilute 10X Permeabilization buffer (Miltenyi Biotec Inc, Earhart Avenue, CA, USA) with distilled water to 1:10 concentration

Table 2 Antibodies used for Flow Cytometry

Antigen	Conjugate	Dilution	Amount (μL)	Isotype	Manufacturer
CD3	APC	1:11	10	IgG2a	Miltenyi Inc
CD4	FITC	1:11	10	IgG2a	Miltenyi Inc
CD8	FITC	1:11	10	IgG2a	Miltenyi Inc
CD19	APC	1:11	10	IgG1	Miltenyi Inc
CD25	PE	1:11	10	IgG2b	Miltenyi Inc
CD127	PE	1:10	20	IgG1	eBioscience
FOXP3	APC	1:11	10	Mouse IgG1	Miltenyi Inc
Isotype control	FITC	1:11	10	Mouse IgG2a	Miltenyi Inc
Isotype control	APC	1:11	10	Mouse IgG1	Miltenyi Inc
Isotype control	AAPC	1:11	10	Mouse IgG2a	Miltenyi Inc
Isotype control	PE	1:11	10	Mouse IgG2b	Miltenyi Inc
Isotype control	PE	1:11	20	Mouse IgG1	Miltenyi Inc

2.2.2 Methods

2.2.2.1 Separation of peripheral blood mononuclear cells from fresh peripheral blood

Twenty mLs of fresh peripheral blood were added to the same volume of RPMI (Gibco) in a 50mL falcon tube. Approximately 5mLs of RPMI and peripheral blood were layered onto 5mLs of lymphoprep (Axis-Shield, Oslo, Norway) in 15mL falcon tubes. The tubes were centrifuged at 524g for 25 minutes at 19 degrees centigrade (˚C). The monolayer was removed from each tube and placed in a 50mL falcon tube. Washing buffer (sterile RPMI with fetal calf serum) was added to a total volume of 50mLs. This was centrifuged at 393g for 10 minutes. The supernatant was discarded and the cells resuspended in 5mLs of washing buffer. A cell count and viability assessment was performed using Trypan Blue (Sigma, Ayrshire, UK). 5μL of cells

were added to 5μL of trypan blue (1:2 dilution) (Sigma) and 10μL added to the haemocytometer chamber. The number of live (white) and dead (blue) cells was counted in 25 squares. Following counting of the cells cell viability and concentration were calculated.

Cell viability: number of unstained cells/ total number of cells and multiplied by 100 to give percentage
Cell concentration (cell/mL) number of cells x dilution factor x 10,000

Aliquots of 1 million cells were removed for flow cytometry analysis at this point and stored on ice.

2.2.2.2 Thawing of cryopreserved cells

The vial was removed from liquid nitrogen and thawed immediately in a water bath at 37˚C and transferred to an empty sterile 15mL Falcon tube. RPMI medium (Gibco) was added dropwise to a volume of 10mL. The tube was centrifuged at 393g for 10 minutes at room temperature. The supernatant was removed and the pellet resuspended in 5mLs of RPMI (Gibco). Approximately 5mLs of RPMI and thawed cells were layered onto 5mLs of lymphoprep (Axis-Shield, Oslo, Norway) in 15mL falcon tubes. The tubes were centrifuged at 524g for 25 minutes at 19 degrees centigrade (˚C). The monolayer was removed from each tube and placed in a 50mL falcon tube. Washing buffer (sterile RPMI with fetal calf serum) was added to a total volume of 50mLs. This was centrifuged at 393g for 10 minutes. The supernatant was discarded and the cells resuspended in 5mLs of washing buffer. A cell count and viability assessment was performed using Trypan Blue (Sigma, Ayrshire, UK). 5μL of cells were added to 5μL of trypan blue (1:2 dilution) (Sigma) and 10μL added to the haemocytometer chamber. The number of live (white) and dead (blue) cells was counted in 25 squares. Aliquots of 1 million cells were removed for flow cytometry analysis at this point and stored on ice.

2.2.2.3 Surface antibody labelling

Aliquots of 1 million cells were placed on ice for flow cytometry analysis. One mL of degassed buffer was added to each aliquot and the samples centrifuged at 393g for 10 minutes at 4-8ºC. Ten μL of each required antibody was then added to the

appropriate tubes and the samples incubated for 10 minutes at 4ºC. The cells were then washed in degassed buffer and centrifuged at 393g for 10 minutes at 4ºC. The supernatant was removed and the cells resuspended in 500µL of degassed buffer.

2.2.2.4 Intracellular labelling

After surface antigen labelling the cells were centrifuged for 10 minutes at 393g and 4-8ºC. The supernatant was removed and up to 1 million cells were resuspended in 1mL of cold Fixation/Permeabilization Solution. The cells were mixed well and incubated for 30 mins in the dark at 4-8ºC. One million cells were washed with 1mL of cold buffer and centrifuged for 5mins at 393g at 4-8ºC and the supernatant was removed. The cells were rewashed in 1mL of cold permeabilization buffer and centrifuged for 5mins at 393g and 4-8ºC. The cells were resuspended in 80µL of cold 1X permeabilization buffer. Then 20µL of FcR Blocking reagent was added, mixed well and the cells refrigerated at 4-8ºC for 5 minutes. The 10µL of FOXP3 antibody were added and incubated in the dark for 30 minutes at 4-8ºC. The cells were then washed with 1mL of cold permeabilization buffer and centrifuged for 5 minutes at 393g at 4-8ºC and the supernatant removed completely. The cell pellet was resuspended in 500µL of buffer.

2.2.2.5 Flow Cytometry and analysis

Flow cytometry was performed on a LSR cytometer (BD Biosciences, Cowley, Oxford). FlowJo Software version 8.1 (Tree Star) was used for analysis. The initial voltage settings were obtained using an unlabelled cell sample. The unlabelled cell sample was also used to draw a gate over the lymphocyte region as determined by forward (FSC) and side scatter (SSC). Compensation levels between FL1 (FITC) and FL2 (PE) were set using cell samples stained with a single fluorochrome.

2.3 Materials and method for immunocytochemistry

2.3.1 Materials

Citrate Buffer for Antigen Retrieval

50ml Antigen unmasking solution (H-3300) (Vector Laboratories) was added 5 litres (L) distilled water, mixed and the pH checked to ensure it was within the range of 6.0-6.5

Pronase antigen retrieval

50 milligrams (mg) of pronase (p5147) was added to 50 millilitres (mL) phosphate buffer

1% Bovine Serum Albumin (BSA) With Azide

2 grams (g) Bovine Serum Albumin was added to 200mL Phosphate buffer and 0.2g sodium azide and dissolved at room temperature. The pH was checked to ensure it was between 7.4-7.6

0.1% Tween Buffer

500 microlitres (µl) Polyoxyethylene Sorbitan Monolaurate (Tween 20) was added to 500mL Phosphate buffer

Phosphate Buffer

72.5g Di-sodium hydrogen orthophosphate (Sorensen's salt) and 11.5g Sodium dihydrogen orthophosphate were added to 1L distilled water and dissolved using the hot plate. The solution was made up to 5L with distilled water and the pH checked to ensure it was pH 7.6

Vector Elite ABC Kit (PK6100, Vector Laboratories, Peterborough, UK)

20µl from bottle A and 20µl from bottle B were mixed with 1000µl of Phosphate buffer and allowed to stand for 30 minutes before use.

Diaminobenzidine (DAB) solution

One Kemtec DAB tablet was dissolved in 10mls of distilled water and allowed to stand for 15 minutes.

2.3.2 Method

Paraffin embedded 4µm sections were cut and placed on TESPA (3-aminopropyl-triethoxysilane) coated slides. The slides were dewaxed by immersion twice for 5 minutes in each xylene and were then placed in absolute alcohol for 2 minutes. Endogenous peroxidase activity was blocked by immersion in 400mL of Industrial Methylated Spirits and 8mL of Hydrogen peroxide. The slides were then taken to tap water. Antigen retrieval was performed using one of the three methods described

below and the slides washed in tap water and 0.1% Tween buffer (2 x 2 mins). The primary antibody was applied at the appropriate dilution for the time recommended by the manufacturer. The primary antibody was diluted with 1% BSA and azide. The sections were then washed with 0.1% Tween buffer (2 x 1 minutes) and the secondary antibody of biotinylated universal antibody applied at a dilution of 1:200 using 1% BSA with azide as the diluent for thirty minutes. The third layer is then made up using the Vector Elite ABC Kit. The sections were washed in 0.1% Tween buffer (2 x 1 minute) and the Vector Elite ABC kit applied for 20 minutes. The diaminobenzidine solution was then prepared. The sections were washed in 0.1% Tween buffer (3 x 1 minute). The diaminobenizidine solution was then activated; for every milliliter (mL) of DAB solution 10µl of 3% hydrogen peroxide was added to activate. The activated DAB solution was applied and left for 10 minutes and the sections were then washed in tap water. The slides were stained with haematoxylin for five minutes to highlight the nuclei and were then rinsed in tap water. The slides were placed in acid alcohol for 2 seconds and the placed in running tap water for 5 minutes. The slides were then dehydrated and coverslipped.

2.3.3 Antigen retrieval using pressure cooking antigen retrieval method

The hotplate was switched on and set to full heat, 3 litres of Vector Antigen Unmasking Solution (H-3300, Vector Laboratories, Peterborough, UK) was placed in the pressure cooker and the lid replaced. The solution was warmed until boiling and then removed from the heat source and the slides placed in the liquid ensuring they were completely covered and the lid closed. The cooker was placed back on the heat source and the pressure selector set to level 2. The solution was allowed to reheat and heating continued until a steady flow of steam was produced from the outlet valve. The timer was set for 10 minutes when a steady flow of steam is produced from the outlet valve. When finished the pressure was released gradually by slowly reducing the pressure selector. Once the pressure selector dropped, the lid was removed and tap water added to the cooker and the slides allowed to stand for 5 minutes. The slides were then removed and placed immediately in tap water. The slides were then placed in phosphate buffered saline (PBS) ready for application of the antibody.

2.3.4 Antigen retrieval using pronase

The sections were placed in the pronase solution at 37˚C for 15 minutes.

2.3.5 Double immunocytochemistry

First primary antibody: the slides were de-paraffinised by immersion in xylene twice for five minutes each and were dehydrated through a series of alcohols twice for three minutes. Endogenous peroxidase activity was blocked by immersion in 400mL of Industrial Methylated Spirits and 8mL of Hydrogen peroxide. The slides were then rinsed in running tap water. The relevant antigen retrieval technique for the antigen was then performed. The slides were rinsed in running tap water and transferred to wash buffer (TBS-T). The slides were incubated in protein blocking solution (5% normal horse serum) for 20 minutes. The excess blocking solution was tipped off and without rinsing the slides were incubated with the primary antibody for 40 minutes to 1 hour at an appropriate dilution for the antibody. The sections were washed in buffer twice for two minutes each and the slides were then incubated with the secondary antibody (biotinylated anti-mouse IgG 1:200) for 30 minutes. The sections were then washed in buffer twice for two minutes. The ABC horseradish peroxidase reagent was applied and incubated for twenty minutes. The slides were then incubated with DAB solution for ten minutes and transferred to running tap water for two minutes to stop the reaction. The sections were then washed twice for two minutes in buffer.

Second primary antibody: the ready-to-use double stain blocking solution (EnVision™ double stain system)(Dako UK Ltd, Ely, Cambs) was applied for three minutes. The slides were then incubated with ready to use avidin blocking solution (Vector Laboratories Inc, California) for fifteen minutes and were rinsed briefly with wash buffer. The slides were then incubated with biotin blocking solution (Vector Laboratories Inc, California) for fifteen minutes and they were then washed twice for two minutes. The slides were incubated with protein blocking solution for twenty minutes, the excess tipped off and slides incubated with the primary antibody diluted in protein blocking solution for 40 minutes to an hour at an appropriate dilution for the antibody. The slides were then washed in wash buffer twice for two minutes and the incubated with the secondary antibody for 10 minutes (biotinylated anti-mouse IgG 1:200). The slides were then washed twice with wash buffer for two minutes. The ABC-alkaline phosphatase (20µl/ml of both A and B in PBS) was applied for 30

minutes and the slides were then washed twice in wash buffer for two minutes. The fast Red solution was prepared by mixing 100 parts of Permanent Red-Substrate Buffer with 1 part of Permanent Red Chromogen (Dako EnVision™ double stain system) (Dako UK Ltd, Ely, Cambs) and incubated for twenty minutes. The slides were stained with haemtoxylin for three minutes. The slides were allowed to air dry and were coverslipped using an aqueous mountant.

2.4 Materials and method for immunofluorescence Vector M.O.M immunodetection kit (Vector Laboratories, Inc, California)

2.4.1 Materials

M.O.M Diluent
600µl of Protein Concentrate stock solution to 7.5ml of Phosphate Buffered Saline (PBS)

M.O.M Biotinylated Anti-Mouse IgG Reagent
Add 10µl of stock solution to 2.5ml of M.O.M diluent

Fluorescein Avidin DCS
Add 40µl of stock solution to 2.5ml of PBS

Texas Red Avidin DCS
Add 40µl of stock solution to 2.5ml of PBS

2.4.2 Method

Paraffin embedded 4µm sections were cut and placed on TESPA coated slides, dewaxed and blocked with hydrogen peroxide/methanol solution. Sections were incubated for 5 minutes in working solution of M.O.M diluent to block protein. Excess M.O.M diluent was tipped off the sections and the primary antibody diluted in M.O.M diluent to the appropriate concentration. The sections were incubated according to recommendations of the manufacturer. The sections were washed in 0.1% Tween buffer twice for two minutes. The secondary antibody, M.O.M Biotinylated Anti-Mouse IgG reagent was applied to the sections and incubated for

20 minutes. The sections were washed in 0.1% Tween buffer twice for 5 minutes each. The Fluorescein avidin conjugate was applied to the sections for 5 minutes; the sections were then washed twice for 5 minutes each with 0.1% Tween buffer. The sections were incubated with avidin solution for 15 minutes, rinsed with 0.1% Tween buffer and then incubated in biotin solution for 15 minutes. The sections were then washed with 0.1% Tween buffer twice for two minutes. The sections were incubated in M.O.M diluent for five minutes, the excess diluent was then tipped off and the secondary antibody diluted to the appropriate concentration. The sections were then washed twice for two minutes in 0.1% Tween buffer. The secondary antibody of M.O.M biotinylated ant-mouse IgG reagent was applied to the slide and incubated for 20 minutes. The sections were then washed twice for two minutes with 0.1% Tween buffer and the Texas Red avidin applied at a concentration of 1:200 and incubated for 10 minutes. The sections were then washed in 0.1% Tween buffer twice for five minutes and then mounted using Vectashield mounting media.

2.5 Tissue microarray construction

2.5.1 Preparation of the Donor Blocks and Slides

A fresh H&E slide was obtained from each donor block and used as a guide to select the region for sampling. The appropriate area was marked on the H&E slide and donor blocks aligned with the appropriate marked H&E slides. Donor blocks were required to be at least 1mm in thickness.

2.5.2 Preparation of the Recipient (Array) Block

Embedding paraffin wax was melted and poured into the mold. A cassette was placed on top of the melted paraffin until cooled. The block was checked to ensure the surface was flat and no air bubbles were trapped within the block. The recipient blocks were at least 5mm in thickness.

2.5.3 Array Design

The array pattern was planned prior to coring tissue encompassing the number of samples to be arrayed. An asymmetrical pattern was constructed to aid orientation before analysis. Punch size of 1.0mm diameter was used with spacing of 2.0mm between the cores and three cores from each sample arrayed. Control appendix and tonsil tissue was included in the array.

2.5.4 Array Construction

The recipient block was inserted into the holder and the clamping screws tightened to prevent slippage of the block. The block within the holder was placed against the location curbs, which is maintained in place by magnets. The depth stop was adjusted to ensure the punch stopped at the correct depth and a hole cored in the empty recipient block using the smaller punch core. At this point the micrometer was set to zero. Release of the paraffin core was aided by rotating the punch by 45°. The donor block bridge was placed over the recipient block holder and the larger punch swiveled into sampling position. The marked H&E slide was aligned with the corresponding tissue block. When correctly aligned remove the H&E slide was removed and the punch pushed downwards to core the sample. Once the sample had been obtained, the bridge was removed and the punch pushed downwards until the tip reached the top of the hole in the recipient array block. In this position the stylet was used to push the tissue core into the hole in the recipient block created by the smaller punch.

2.5.5 Sectioning the Array Block

Prior to sectioning the block was placed in a chamber heated to 37°c for 10-15 minutes. This enabled improved adherence of the tissue cores to the recipient block. Once warmed and slightly malleable a glass slide was used to level the wax surface. A standard microtome technique was used to section the array block.

2.6 Materials and Methods for Western Blotting

2.6.1 Materials

Extraction/Labelling buffer

10mLS of extraction/labelling buffer (Clontech Lab Inc.) and 1 tablet of protease inhibitor cocktail (Roche Diagnostics)

BCA™ Reagent A (Pierce, Rockford, IL)

Sodium carbonate, sodium bicoarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide

BCA™ Reagent B (Pierce, Rockford, IL)

4% cupric sulfate

BCA™ Working reagent (Pierce, Rockford, IL)
Mix 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B. On addition of reagent B to reagent A, turbidity was observed which disappeared to leave a clear green working reagent

Running buffer
950mLs of deionised water and 50mLs of Novex® Tris-Acetate SDS Running Buffer 20X (Invitrogen, Carlsbad, CA, USA)

Transfer buffer
950mLs of deionised water and 50mLs of NuPAGE® Transfer Buffer 20X (Invitrogen, Carlsbad, CA, USA)

Washing buffer (TBS-Tween)
900mLs of deionised water and 100mLs of 10 x TBS (24.2 grams of Trizma base, 80 grams of NaCl made up to 1 litre with deionised water, pH to 7.6 with concentrated hydrochloric acid) and 100µL of Polyoxyethylene Sorbitan Monolaurate (Tween 20)

Blocking buffer
10g Marvel milk powder and 200mLs of 0.1% TBS-Tween

2.6.2 Methods
2.6.2.1 Cell lysis and preparation of protein homogenate
Once the cells were 80% confluent the media was removed and 10mLs of 0.05% trypsin was added to the flask. This was incubated for 5 minutes at 4˚C and then tapped to ensure all the cells had detached. Fifteen mLs of media was then added and the cell suspension was transferred to a falcon tube and centrifuged for 5 minutes at 393g and 19˚C. The supernatant was discarded and the cell pellet resuspended in 25 mLs of PBS. The cell suspension was centrifuged for 5 minutes at 393g and 19˚C and the supernatant removed completely. The cell pellet was placed in a -80˚C freezer for 5 minutes and was then resuspended in 200µL of Extraction/labeling buffer (Clontech Lab Inc.) and homogenized. The mixture was transferred to an

eppendorf tube and centrifuged for 10 minutes at 14000rpm (Eppendorf centrifuge 5417R) and 4˚C. The supernatant was transferred to a new eppendorf and stored at -80˚C.

2.6.2.2 Protein quantification using the BCA™ Protein Assay Kit (Pierce, Rockford, IL)

The protein content of each cell lysate was measured using the BCA™ Protein Assay kit which combines the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium with a sensitive colorimetric detection of the Cu^{+1} using a reagent containing bicinchoninic acid (BCA). The end reaction product is composed of two molecules of BCA with a single cuprous ion and absorbs at 562nm which is linear over increasing protein concentrations. A series of proteins of known concentrations are prepared and assayed alongside the samples before the concentration of the sample is determined based on the standard curve.

Table 3 Preparation of Diluted Albumin (BSA) Standards (adapted from BCA™ Protein Assay Kit datasheet)

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA concentration
A	0	300µl of Stock	2,000µg/ml
B	125µL	375µl of Stock	1,500µg/ml
C	325µL	325µl of Stock	1,000µg/ml
D	175µL	175µl of vial B dilution	750µg/ml
E	325µL	325µl of vial C dilution	500µg/ml
F	325µL	325µl of vial E dilution	250µg/ml
G	325µL	325µl of vial F dilution	125µg/ml
H	400µL	100µl of vial G dilution	25µg/ml
I	400µL	0	0µg/ml = Blank

Twenty five microlites of each standard or sample was pipetted into a microplate well in duplicate and 200µl of the working reagent added to each well and the plate was mixed on a plate shaker for 30 seconds. The plate was covered and incubated at 37˚C for 30 minutes. The plate was cooled to room temperature and the absorbance measured at 562nm on a plate reader.

2.6.2.3 Protein preparation for sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE)

15µg of cellular lysate as determined above was added to reducing agent and buffer. The volume of the cellular lysate was divided by 4 to obtain the volume of buffer required and by ten to calculate the volume of reducing agent required. The samples were then heated to 95˚C for 10 minutes and cooled on ice.

2.6.2.4 Determination of relative molecular weight by SDS-PAGE electrophoresis

Novex® Sharp Pre-stained Protein Standard (Invitrogen, Carlsbad, CA, USA) was used to visualize the protein molecular weight. The standard consists of 12 pre-stained protein bands ranging from 3.5 -260kDa. Ten microlitres of standard was run alongside the samples on each gel to allow the relative size of the protein of interest to be determined.

2.6.2.5 Western Blotting using XCell SureLock™ Mini-Cell *(Invitrogen, Carlsbad, CA, USA)*

A 1.0mm ten well NuPAGE® 3-8% Tris-Acetate gel (Invitrogen, Carlsbad, CA, USA) was placed in the XCell *SureLock*™ Mini-Cell having removed the packaging and the tape from the gel cassette. The comb was removed and the wells gently pipetted with running buffer. The buffer core containing the electrodes and terminals was inserted into the chamber of the XCell *SureLock*™ Mini-Cell. The gel cassettes were then inserted into the chamber, one cassette was placed behind the core and the other in front of the core. The Gel Tension Wedge was inserted into the chamber and the lever closed to push the gels firmly against the buffer core. The buffer chamber was then filled with running buffer and checked to endure no leakage occurred. The Novex® Sharp Pre-stained Protein Standard (Invitrogen, Carlsbad, CA, USA) was loaded into the first well of each gel and the cellular lysates were loaded into the

other wells. Sample buffer was loaded into any unused wells. The lower buffer chamber was filled with 600mL of running buffer and electrophoresis of the gel was performed at a constant voltage of 200V for 35 minutes.

2.6.2.6 Electroblotting of separated protein in the SDS-PAGE gel onto Amersham Hybond ECL nitrocellulose membrane using the XCell II™ Blot module (Invitrogen**,** Carlsbad, CA, USA)

After electrophoresis the protein in the gel was transferred to the surface of a thin support membrane. This was accomplished by placing the gel next to the membrane and they were placed in a voltage gradient perpendicular to the gel. Negatively charged molecules migrated from the gel towards the positive electrode and deposited on the membrane. The gels were removed from the plastic plate and electroblotted onto nitrocellulose membranes (Amersham Hybond ECL Nitrocellulose membrane, GE Healthcare Ltd, Buckinghamshire, UK)). The blotting pads were soaked in transfer buffer until saturated and air bubbles were removed by squeezing the blotting pads whilst submerged in transfer buffer. The gel was then placed on a piece of filter paper on top of the blotting pads and the membrane was layered on top of the gel and air bubbles removed by rolling a pipette over the membrane. The final blotting pad was placed on top of the membrane the stack transferred into the cathode core of the blot module with the gel being closest to the cathode. The anode core was then placed on top of the stack. The gel membrane sandwich and blotting pads in the cathode core were placed in the XCell II™ Blot module. The gel membrane sandwich was covered with transfer buffer. The gels were electroblotted at a constant voltage of 18V for 2 hours at room temperature.

2.6.2.7 Antibody staining of western blots

Determination of protein expression was assessed by antibody staining. Membranes were also stained with a GAPDH antibody to ensure equal loading of the samples per lane. Membranes were blocked with 5% (w/v) milk (Marvel) and TBST (0.1%) for 1 hour. The membranes were washed twice in TBST for 6 minutes. The primary antibody was then applied overnight at 4°C. The membranes were washed twice again for 6 minutes and the secondary horseradish peroxidase-conjugated applied.

2.6.2.8 Antibodies used for immunoblotting and dilutions used

The following primary antibodies were used in Western blot experiments: monoclonal Mouse Anti-Human p53 Protein Clone DO-7 (Dako Ltd, Cambridge, UK) (1: 1000), monoclonal anti-human murine double minute 2 (MDM2) gene product Clone 1B10 (Novocastra, Newcastle, UK) (1:500) and anti-human mouse anti-$p21^{Cip1/WAF1}$ Clone EA10 (Zymed Laboratories, South San Francisco, California, USA) (1:500) and anti-human rabbit polyclonal Clusterin-α/β antibody Clone H-330 (Santa Cruz Biotechnology, Inc, USA)(1:200). A horseradish peroxidase-conjugated polyclonal rabbit anti-mouse IgG_1 was used as secondary antibody for mouse primary antibodies at 1:2000 (Dako Ltd, Cambridge, UK) and a horseradish peroxidase-conjugated polyclonal goat anti-rabbit IgG_1 was used as secondary antibody for rabbit primary antibodies at 1:2000 (Dako Ltd, Cambridge, UK). Excess secondary antibody was removed by a further four washes of 15 minutes each in TBST.

2.6.2.9 Visualisation of protein bands using enhanced chemiluminescence

The protein bands were visualised using an enhanced chemiluminescence (ECL-plus) (GE Healthcare Ltd, Buckinghamshire, UK) visualisation system. Hydrogen peroxide catalyses the oxidation of luminal in alkaline conditions and the luminal decays to ground state via a light emitting pathway. Membranes were incubated with ECL for 2 minutes before the excess was blotted away and exposed to blue light sensitive autoradiography film, Hyperfilm™ ECL (GE healthcare Ltd, Buckinghamshire, UK) for 5 seconds to 30 mins depending upon the strength of the bands.

2.6.2.10 Control antibody to ensure equal loading of wells

After the initial protein bands were visualized, the membranes were reblocked using 5% (w/v) milk (Marvel) and TBST (0.1%) for 1 hour. The membranes were washed twice in TBST for 6 minutes. The GAPDH antibody (Cell Signaling) was then applied at a dilution of 1:2000 at room temperature for one hour. The membranes were washed twice again for 6 minutes and the secondary horseradish peroxidase-conjugated applied for one hour at room temperature. Excess secondary antibody was removed by a further four washes of 15 minutes each in TBST. The protein bands were visualised using an enhanced chemiluminescence (ECL-plus) (GE

Healthcare Ltd, Buckinghamshire, UK) visualisation system.

CHAPTER THREE:

The role of p53, MDM2 and WAF-1 in transformation of follicular lymphoma to diffuse large B cell lymphoma

3.1 Introduction

Although follicular lymphoma has a median survival from diagnosis of between eight to ten years (Gallagher CJ et al. 1986), a substantial proportion of these patients will transform to a high grade more aggressive lymphoma usually diffuse large B cell lymphoma. (Hubbard SM et al. 1982; Acker B et al. 1983; Bastion Y et al. 1997, Montoto S et al. 2007). These patients typically respond poorly to therapy and have a poor prognosis (Gallagher CJ et al. 1986; Bastion Y et al. 1997). Although many potential oncogenic events have been linked to the process of transformation including mutation of the *TP*53 tumour suppressor gene, the molecular mechanisms and their relationship to transformation are, at present, poorly understood.

The aim of this study was to identify the frequency of p53 mutation in transformed follicular lymphoma and correlate the timing of its occurrence to the process of transformation. In addition, the use of immunocytochemistry as a surrogate to mutation analysis in identifying p53 mutations was assessed. The protein expression of MDM2 and p21, as targets of wild type p53, was analysed in relation to p53 mutational status to determine if immunocytochemistry is an adequate surrogate for determining genomic mutation.

3.1.1 *TP53*

The *TP*53 gene was initially discovered in 1979 and originally was proposed to be an oncogene (Lane DP, Crawford LV 1979; Linzer DI, Levine AJ 1979). The protein product of the gene was shown to complex with the SV40 T antigen in SV40 transformed cells (Lane DP, Crawford LV 1979; Linzer DI, Levine AJ 1979; Sarnow P, Ho YS, Williams J, Levine AJ 1982). Subsequent work revealed that the normal wild type protein acts as a tumour suppressor gene. Using *TP53* cDNA-genomic hybrid clones it was demonstrated that a mutation at position 135 was required to activate the p53 protein for cooperation with the *ras* oncogene. Additionally, other mutations in *TP53* were able to activate and transform rat embryo cells with the *ras* oncogene suggesting that there is prevention of wild-type p53 function by mutation, which would be in keeping with *TP53* being a tumour suppressor gene (Hinds P, Finlay CA, Levine AJ 1989). DNA clones of wild type *TP53* were able to prevent the transformation of primary rat embryo fibroblasts by E1A, an adenovirus protein which is an oncoprotein, plus *ras* or mutant *TP53* and *ras* activated oncogenes. This

further indicated that p53 can act to block transformation of fibroblasts (Finlay CA, Hinds PW, Levine AJ 1989). The human *TP53* gene is located on chromosome 17p13 (Isobe M et al. 1986). The half life of p53 protein is extended to many hours in transformed cells containing complexes of DNA tumour viruses in comparison to a half life of between 6 and 30 minutes in non transformed cells (Oren M, Maltzman W, Levine AJ 1981). The regulation of the quantity of p53 protein is controlled at both mRNA and protein stability level; regulation of protein degradation is responsible for turnover of the protein (Reich NC, Oren M, Levine AJ 1983). Mutations in *TP*53 which cause structural alteration of the protein product and increased stability of the protein product result in the ability to detect it by immunocytochemical staining (Gannon JV et al. 1990). Analysis of murine *p53* mRNA levels demonstrated the presence of transcripts in both normal and malignant tissue; levels in normal tissue were low but were vastly increased in tumours (Rogel A et al. 1985). Mutations in *TP*53 occur in numerous human tumours; most tumours with allelic deletions of chromosome 17p contain point mutations of *TP53*. These mutations are predominantly located in four hotspots which correspond to the four most conserved regions of the genes. However, allelic deletion is not essential for point mutation to occur. These results suggest that mutations of *TP53* may be important in the development of many malignancies (Nigro JM et al. 1989). The location of the *TP53* gene is a region shown to be a site of recurrent loss in transformation of FL to DLBCL (Hough RE et al. 2001, Goff LK et al. 2000, Martinez-Climent JA et al. 2003). Wild type p53 protein acts to inhibit DNA synthesis at G1 in cells after DNA damage, caused both by pharmacological means or γ irradiation has occurred (Kastan MB et al. 1991; Kuerbitz SJ et al. 1992). Wild type p53 protein also activates apoptotic pathways (Debbas M, White E 1993). Given this major function, it is easy to understand why reduced activity of p53 results in genomic instability and inappropriate survival of damaged cells, essential factors in tumourigenesis.

The ability to control apoptosis is important to the role of *TP*53 as a tumour suppressor gene. A study by Lowe et al. (1994) has shown that the absence of p53 within mouse embryonic fibroblasts immortalized the cell and increased cell growth. In addition, the lack of p53 allowed an oncogene to convert the phenotype of the cells into a tumorigenic one (Lowe SW et al. 1994). Its presence in solid tumours

correlates both with unfavourable prognosis (Isola J et al. 1992; Silvestrini R et al. 1993) and resistance to treatment (Visakorpi T et al. 1992). Mutations of *TP53* are so common in human solid tumour malignancy that their presence may be essential to the development of malignancy. This inference is supported by the observation that even in neoplastic cells with normal *TP53* alleles other mechanisms upstream of *TP53* act to reduce the expression of the protein or alter its function. This includes overexpression of the regulatory protein MDM2 (Momand J et al. 1992; Finlay CA 1993). Mutant *TP53* may act in two different ways to promote neoplastic development, it may complex with wild type normal *TP53* and prevent its normal function, a dominant negative effect (Blagosklonny MV 2000). A second method may be gain of function whereby the mutant protein actively promotes carcinogenesis although the exact mechanisms by which this occurs remain unclear (van Oijen MG, Slootweg PJ 2000). Mutation of *TP53* is a less common occurrence in haematological malignancies than in solid tumours. Analysis of samples from 37 patients with multiple myeloma demonstrated a *TP53* mutation in only one case (Preudhomme C et al. 1992) and 8 out of 112 patients with acute myeloid leukaemia (AML) had a *TP53* mutation (Fenaux P et al. 1992a). Analysis of samples from patients with chronic lymphatic leukaemia (CLL) established mutation in 4 out of 39 cases. All patients with *TP53* mutation had 17p monosomy and the presence of mutation was linked to advanced clinical stage (Fenaux P et al. 1992b).

Although mutation of *TP53* is a common occurrence in many malignancies the exact temporal relationship between acquisition of mutation and development of neoplasia is variable. Oesophageal malignancies have been shown to accumulate *TP53* mutations at an early stage with mutated forms being detected in the metaplastic cells of Barrett's oesophagus. Genetic abnormalities within regions of Barrett oesophagus, which involve *TP*53 can be detected can be present as many as six years prior to the development of malignancy (Barrett MT et al. 1999). In contrast allelic deletions of *TP53* are a later event in the development of gastric carcinoma (Rhyu MG et al. 1994). A theory to explain this surmises that the nature of the stress activating the p53 pathway determines the stage at which mutation occurs. In malignancies where there is a high degree of environmental insult as a contributory cause, for example carcinoma of the lung, the p53 pathway is activated in stressed cells as a defensive mechanism. Damaged cells as a result, undergo apoptosis or repair and consequently

only those cells with mutated *TP53* are able to survive. However, in those malignancies where mutagens are not a direct cause, genetic alterations occur which activate the p53 pathway by oncogenic stress. In cells with normal p53 function, the cell cycle will be halted and only those with mutated *TP53* will survive (Guimaraes DP, Hainaut P 2002).

Chemotherapy regimes aim to prevent proliferation of malignant cells and if possible cause their death. One mechanism of causing cell death is via apoptosis. Observation of tissue morphology of the small intestine in mice showed that the number of cells undergoing apoptosis was increased from that seen in normal proliferating cells after injection of various chemotherapeutic agents. There was no evidence of coagulative necrosis (Searle J et al. 1975). Mouse embryonic fibroblasts deficient in p53 can be sensitised to undergo apoptosis in response to radiation and chemotherapeutic agents by an oncogene (adenovirus E1A gene) but p53 is necessary for this process (Lowe SW et al. 1993). Inactivating mutations of *TP53* may contribute to chemoresistance of some malignant neoplasms. Murine studies have demonstrated that p53 containing tumours treated with radiation or adriamycin showed increased levels of apoptosis and responded better to treatment in comparison to p53 deficient tumours (Lowe SW et al. 1994).

The *TP*53 gene possesses a transcriptional activation sequence, which regulates the expression of many different genes possessing a p53 binding site (Unger T et al. 1992). These include *MDM2* (Barak Y et al. 1993, Chen CY et al. 1994) and $p21^{Waf1/CIP1}$ (El-Diery WS et al.1993).

3.1.2 Mouse double minute-2 protein (MDM2)

MDM2 protein was initially detected in a complex with p53 and inactivates its transactivation function (Momand J et al 1992). MDM2 and p53 act to regulate each other, the transcription of MDM2 is enhanced by wild type p53 (Barak Y et al. 1993). The N-terminal portion of the human MDM2 protein is critical for complex formation with p53 (Chen J, Marechal V, Levine AJ 1993) and the residues important for binding of p53 to mdm2 are the same residues involved in transactivation (Kussie PH et al 1996). This indicates that MDM2 inhibits p53 function by disruption of its interaction with transcription machinery (Chen J,

Marechal V, Levine AJ 1993). MDM2 protein can inhibit all three actions of wild-type and mutated p53 protein; transcriptional activation, repression and mutant protein activation. Thus when MDM2 protein is overproduced, which may result in transformation of a cell or tumour formation, all the functions of p53 are inactivated (Chen J, Lin J, Levine AJ 1995). MDM2 also acts by ubiquitin-mediated proteolysis to target p53 for degradation (Haupt Y et al. 1997). Thus a regulatory loop is formed between the two genes which regulates their trancription (Chen CY et al. 1994). The *MDM2* gene is located on chromosome 12q13-14 and encodes a nuclear protein (Oliner JD et al. 1993). Overexpression of the *MDM2* gene is seen in many human cancers especially sarcomas (Cordon-Cardo C et al. 1994) with varying impact on prognosis. These observations and evidence that MDM2 protein can inactivate p53 mediated transcriptional activation suggested that its ability to promote tumourigenesis is a result of inactivating p53 (Leach FS et al. 1993; Oliner JD et al. 1992). This was subsequently proved when MDM2 was shown to inhibit growth suppression and apoptosis mediated by p53 in tumour derived cells (Chen CY et al. 1994; Chen J et al.1996). In murine models deletion of *Mdm2* results in embryonic lethality which is overcome by the loss of *p53* (de Rozieres S et al 2000).

3.1.3 $p21^{WAF1/CIP1}$

$p21^{WAF1/CIP1}$ is located on chromosome 6p and is a downstream target of wild type p53 which can induce its transcription. It is a cyclin dependent kinase inhibitor involved in regulation of the cell cycle (Harper JW et al. 1993). Activation of p53 as a result of DNA damage causes downstream transcriptional activation of *$p21^{WAF1/CIP1}$*. This then acts to inhibit cyclin dependent kinases and arrests the cell cycle at the G1-S phase. $p21^{WAF1/CIP1}$ also prevents activation of DNA polymerase and thus inhibits DNA replication in S phase (Waga S et al. 1994). Mutant p53 is unable to activate transcription of $p21^{WAF1/CIP1}$ (Dulic V et al. 1994). Immunocytochemically, the distribution of $p21^{WAF1/CIP1}$ expression in normal human adult tissues showed intense nuclear staining in a subset of nuclei in the thyroid, prostate, muscle, kidney, brain, testes and gastrointestinal therapy with very little staining in lymph nodes, liver, spleen, brain, heart and lung. Immunocytochemical expression of $p21^{WAF1/CIP1}$ in DNA damaged human skin (by UV radiation) was nuclear in location and increased as was p53 expression. This was only observed after the skin was treated with UV radiation. Analysis of $p21^{WAF1/CIP1}$ promoter

sequences revealed that this induction was probably mediated by conserved p53 binding sites. Within the colonic epithelium there was a strict topological restriction of $p21^{WAF1/CIP1}$, the Ki67 positive proliferating compartment of epithelial cells ceased near the base of the crypt and became Ki67 negative and $p21^{WAF1/CIP1}$ positive. This effect was lost in colonic tumours suggesting that loss of the topological control may be a feature of neoplastic transformation (El-Diery WS et al. 1995).

3.1.4 Aim of this study

Previous studies have identified mutation of *TP53* and overexpression of the protein at immunocytochemical level in transformed follicular lymphoma (Sander CA et al. 1993, Lo Coco F et al. 1993). Despite small numbers of cases the implication is that *TP53* mutation is acquired prior to transformation to DLBCL, however the exact temporal relationship is uncertain as is the length of latency period between mutation and transformation. In addition, the use of immunocytochemistry as a surrogate for mutation of *TP53* is currently used. This work aimed to assess the correlation between mutation at the gene level and protein overexpression. This may be of increasing importance with the development of p53 directed therapy.

3.2 Material and Methods

3.2.1 Patient Samples

Ethics approval was obtained for this project from the local regional ethics board. Twenty nine patients who were diagnosed with follicular lymphoma (FL) and subsequently transformed to diffuse large B cell lymphoma (DLBCL) were identified and ninety-one lymphoma specimens from these patients retrieved from the archives of St. Bartholomew's Hospital. To be included in this study the presence of a biopsy of FL and a subsequent biopsy confirming transformation to DLBCL under the WHO criteria were required. Where available, additional biopsy samples obtained both pre- and post-transformation, were analysed. Presentation lymph nodes were available in 13 patients, the relatively small number reflecting the fact that many patients are originally diagnosed elsewhere and subsequently referred to the cancer centre at St Bartholomew's Hospital.

3.2.2 *TP53* mutation detection

Genomic DNA was extracted from snap-frozen lymph nodes and the entire coding

sequence of *TP53* was examined. This was carried out by polymerase chain reaction (PCR), fluorescent single strand conformation polymorphism (FSSCP) analysis and direct sequencing. PCR amplification was carried out using Amplitaq Gold polymerase (Applied Biosystems, Warrington, UK) in the manufacturers' buffer, 200µm of each dNTP; 0.5pmol/µl of each primer and 2ng/µl genomic DNA. For FSSCP, the primers were labelled with FAM or HEX and the fluorescent-labelled PCR products were diluted from 1:10 to 1:40 with water according to the yield. 1-2µl of diluted product was mixed with 0.5µl of ROX-500 size standards (Applied Biosystems) and 10.5µl of HI-Deionised Formamide (Applied Biosystems). The samples were denatured at 95°C for 2 minutes and subsequently snap cooled on ice. FSSCP analysis was carried out on a 3100 Genetic Analyser (Applied Biosystems) at 18 and 30°C using 5% Genescan polymer containing 10% glycerol and 1 x TAPS buffer with EDTA. Data was analysed using Genescan 3.7.1 and Genotyper 2.5 software (Applied Biosystems). Two independent observers visually examined each electropherogram trace.

Bidirectional sequencing was carried out using BigDye Terminator chemistry (Applied Biosystems) and the primers for PCR. Electrophoresis was performed on a 310 Genetic Analyser (Applied Biosystems) and the data analysed using Sequence Analysis 3.0 (Applied Biosystems); SeqMan (DNAStar, Madison, WI, USA) and by visual inspection of the electropherograms. Nucleotide number was assigned according to sequence U94788. LOH was examined at five common polymorphic sites: 11827 G>C, 11992 C>A, 11951_11952ins16, 12139 G>C and 17708 A>T. The PowerPlex16 multiplex PCR single tandem repeat (STR) genotyping system (Promega, Southhampton, UK) was performed according to the manufacturer's instructions. The above work was performed with AJ Davies, C Taylor, LK Goff, S Iqbal and D Cuthbert-Heavens.

3.2.3 Validation of monoclonal antibody specificity using Western blotting

The cell lines BT549 (Human Mono Epithelial Breast Infiltrating Carcinoma) and CRL2366 (Human Mono Fibroblast Glioblastoma) (both obtained from Cell Services, CRUK) were cultured in RPMI with 10% fetal calf serum (Gibco) and 1% streptomycin/penicillin (Clare Hall, CRUK). The protein from the cell lines was extracted using 200 µL of extraction /labelling buffer (Clontech Lab Inc.) and the

amount of protein analysed using the BCA™ Protein Assay Kit (Pierce, Rockford, IL). Fifteen micrograms of protein was suspended with loading buffer and reducing agent and denatured by heating at 95˚C for ten minutes. The mixture was loaded onto the gel and run for 35 minutes at 200V. The gel was transferred to a nitrocellulose membrane and blocked for one hour with blocking solution. The primary antibody was incubated overnight at 4˚C at dilutions of 1:1000 for p53, 1:500 for MDM2 and 1:500 for p21. The secondary antibody was applied at a dilution of 1:2000 for two hours.

3.2.4 Immunocytochemistry

Paraffin blocks from 77 of the 91 samples analysed for *TP53* mutation were available for immunocytochemical analysis. Both FL and DLBCL samples were available in 20 out of the 29 patients.

3.2.4.1 P53 Immunocytochemistry

The antibody used to perform immunocytochemistry was monoclonal Mouse Anti-Human p53 Protein Clone DO-7 (Dako, Glostrup, Denmark). Tissues processed by standards protocols had 4μm sections were cut and placed on TESPA (3-aminopropyl-triethoxysilane) coated slides, were then dewaxed with xylene and rehydrated through graded alcohols and non-specific binding blocked with hydrogen peroxide/methanol solution. Antigen retrieval was performed overnight at 60°c using 50mLs of antigen unmasking solution (H-3300, Vector Laboratories, Peterborough, UK). Primary antibody was diluted and incubated at 1:100 for 40 minutes. Sections were then stained using the Vector Elite ABC kit (PK6100, Vector Laboratories, Peterborough, UK) and chromogen diaminobenzidine (Biostat, Stockport, UK).

3.2.4.2 MDM2 Immunocytochemistry

The antibody used was monoclonal anti-human murine double minute 2 (MDM2) gene product Clone 1B10 (Novocastra, Newcastle, UK). This was performed exactly as described above except antigen retrieval which was performed using pressure cooking antigen retrieval method (Norton AJ et al. 1994) using Vector Antigen Unmasking Solution (H-3300, Vector Laboratories, Peterborough, UK). The primary antibody was diluted and incubated at 1:100 overnight.

3.2.4.3 WAF-1 (p21) Immunocytochemistry

The antibody used was monoclonal anti-human mouse anti-$p21^{Cip1/WAF1}$ Clone EA10 (Zymed Laboratories, South San Francisco, California, USA). This was performed as described above using the pressure cooking method of antigen retrieval (Norton AJ et al. 1994) and application of the primary antibody at a dilution of 1:100 overnight at 4ºC.

All biopsies from every patient were stained with each monoclonal antibody and for each antibody an individual negative control was also produced.

3.2.5 Immunocytochemical Scoring

For each antibody the staining was assessed to ensure appropriate staining was seen in accordance with the monoclonal antibody datasheet and published literature. The percentage of positive cells was recorded for each individual antibody. Only nuclear staining was considered positive for all three antibodies. In the cases of FL the entire section was initially scanned at low power (magnification x5) and the number of positive cells within ten complete follicles subsequently scored at x40 magnification. In the cases of DLBCL the entire section was initially scanned at low power (magnification x5) and the number of positive cells scored within ten fields at x40 magnification. If more than one block of tissue was available for scoring from each sample the median of the scores was calculated.

3.2.6 Statistical Analysis

Differences between the immunocytochemical staining within subgroups were analysed using the nonparametric Mann-Whitney U test with significance set at $p<0.05$. Differences in subgroup survival were assessed using the log-rank test. Survival data was analysed using Kaplan Meier method.

3.3 Results

The presence of *TP*53 mutations at a genomic and protein level were examined in biopsies from the 29 patients with FL who subsequently transformed to DLBCL Ninety one tissue samples were studied for mutations at the genomic level and seventy seven samples were analysed for proteomic mutations using immunocytochemistry. The prevalence of *TP*53 mutation was assessed and correlated with survival. The genomic mutation status was correlated with tissue expression of p53, MDM2 and p21 proteins.

3.3.1 Patient Characteristics

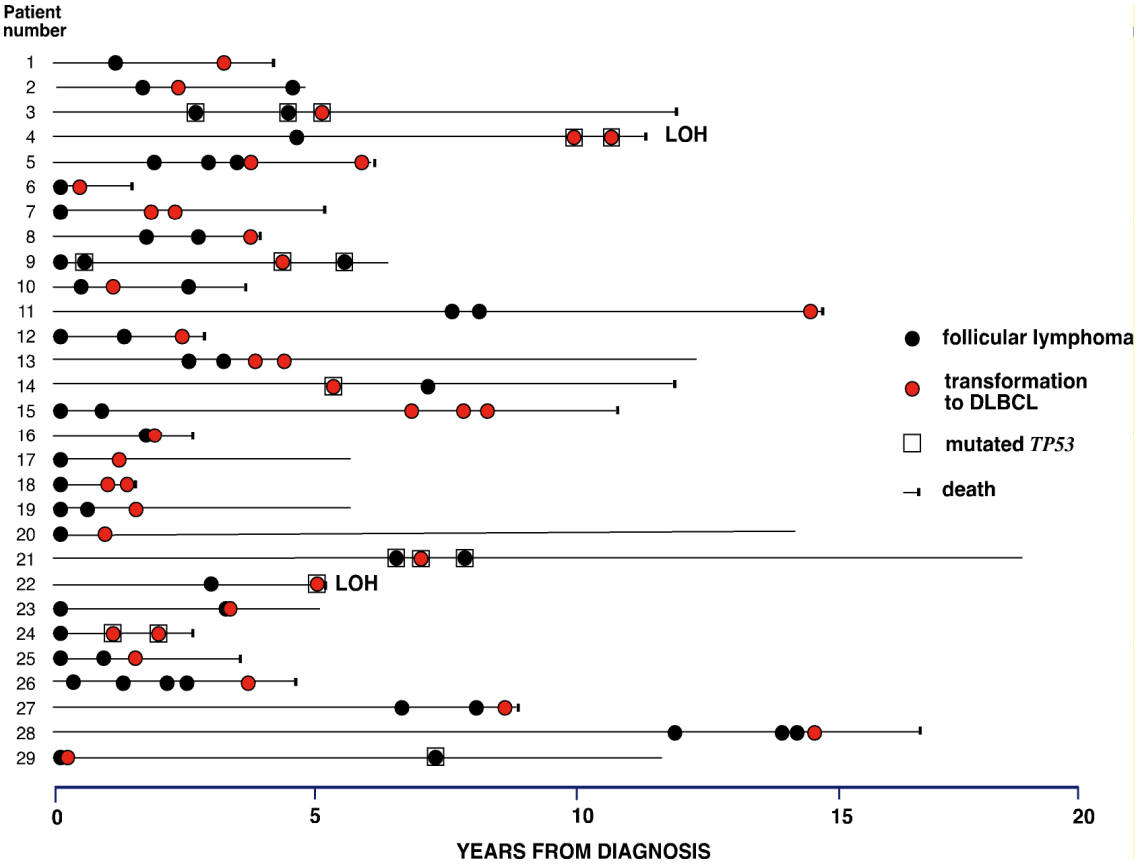
The median age of the patients at transformation was 52 years with a range from 27 years to 73 years. After presentation, transformation occurred after a median of 3.8 years (range 11 weeks to 15.2 years) and after a median of 3 prior therapies (range 0-13).

3.3.2 Mutational Analysis of *TP53*

The entire coding sequence of *TP*53 and the first non-coding exon were analysed. This did not demonstrate any mutations in the 13 biopsies obtained at initial diagnosis of FL.

All twenty nine patients suffered a recurrence of lymphoma and ten mutations were detected in eight of these patients (28%). Five of these mutations were missense mutations, which resulted in a single amino acid substitution. The remaining mutations were made up of two nonsense mutations, a branch site mutation, a splice mutation and an insertion. All of the mutations were detected within exons 5-7 despite screening of the entire coding sequence. The identified mutations were observed in FL samples in 4/29 (14%) patients and 7/29 (24%) transformed FL (t-FL) patients (**Figure 20**) (p=0.1).

Figure 20 Diagrammatic illustration of serial biopsies obtained from 29 patients with FL which transformed to DLBCL analysed for TP53 mutations (Davies AJ et al. 2005 with permission).



In five patients mutations were present in the t-FL sample but not in the preceding FL sample. Three of these patients acquired the mutation at the time of transformation and all underwent an aggressive clinical course with poor outcome. Of the eight patients with mutations, six of these suffered recurrence of their lymphoma after successful treatment for t-FL. In four of these patients an identical mutation was detected, two of these patients relapsed with FL and two relapsed with t-FL. One patient was treated to complete remission for t-FL and subsequently attained a mutation in a FL sample seven years later. In the final patient the mutation was eradicated and the recurrent FL sample contained *wtTP53* suggesting relapse from a clone that had not undergone transformation.

In three patients, mutation of *TP53* was observed in a FL sample prior to transformation. The time period between detection of the mutation and transformation to DLBCL differed and was 6 months, 2.5 years and 4 years respectively. Thus the presence of a mutation in *TP*53 does not necessarily indicate that transformation to DLBCL is imminent.

No significant difference in survival from diagnosis or survival from time of transformation was detected between those patients with a *TP*53 mutation and those without mutation ($p=0.19$ and $p=0.45$ respectively).

3.3.3 Validation of antibody specificity by Western Blotting

The specificity of the antibodies used for immunocytochemistry was validated on BT549 (Human Mono Epithelial Breast Infiltrating Carcinoma) and CRL2366 (Human Mono Fibroblast Glioblastoma). These cells lines were recommended by the manufacturer of the antibody for western blotting.

Figure 21 Western Blot of the BT549 and CRL2366 cell lines demonstrating a strong band at 53Kda in the BT549 lane and a fainter band in CRL2366 using the monoclonal antibody to p53 (Mouse Anti-Human p53 Protein Clone DO-7 (Dako, Glostrup, Denmark)).

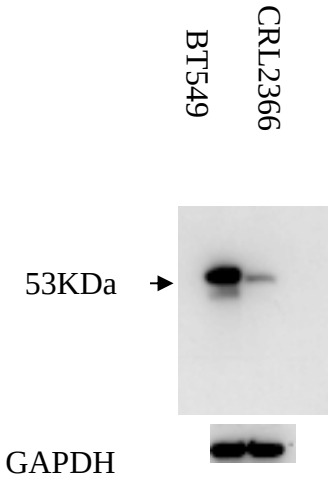
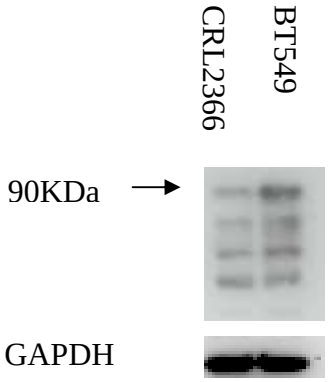
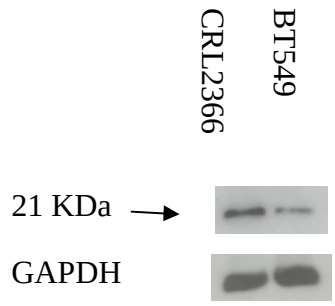


Figure 22 Western Blot of the BT549 and CRL2366 cell lines demonstrating five bands at 90 KDa, 85KDa, 76KDa, 74KDa and 57-58KDa in both cell lines using the monoclonal antibody to MDM2 (monoclonal anti-human murine double minute 2 (MDM2) gene product Clone 1B10 (Novocastra, Newcastle, UK))



The presence of alternatively spliced MDM2 proteins has been previously reported. Within cell cultures, polypeptides with molecular weights of 90KDa, 85 KDa, 76KDa, 74KDa and 57-58KDa have been detected (Olson DC et al. 1993). The presence of different splice forms has also been detected in epithelial tumours including breast carcinoma (Bueso-Ramos CE et al. 1996) and bronchogenic carcinoma (Gorgoulis VG et al. 1996).

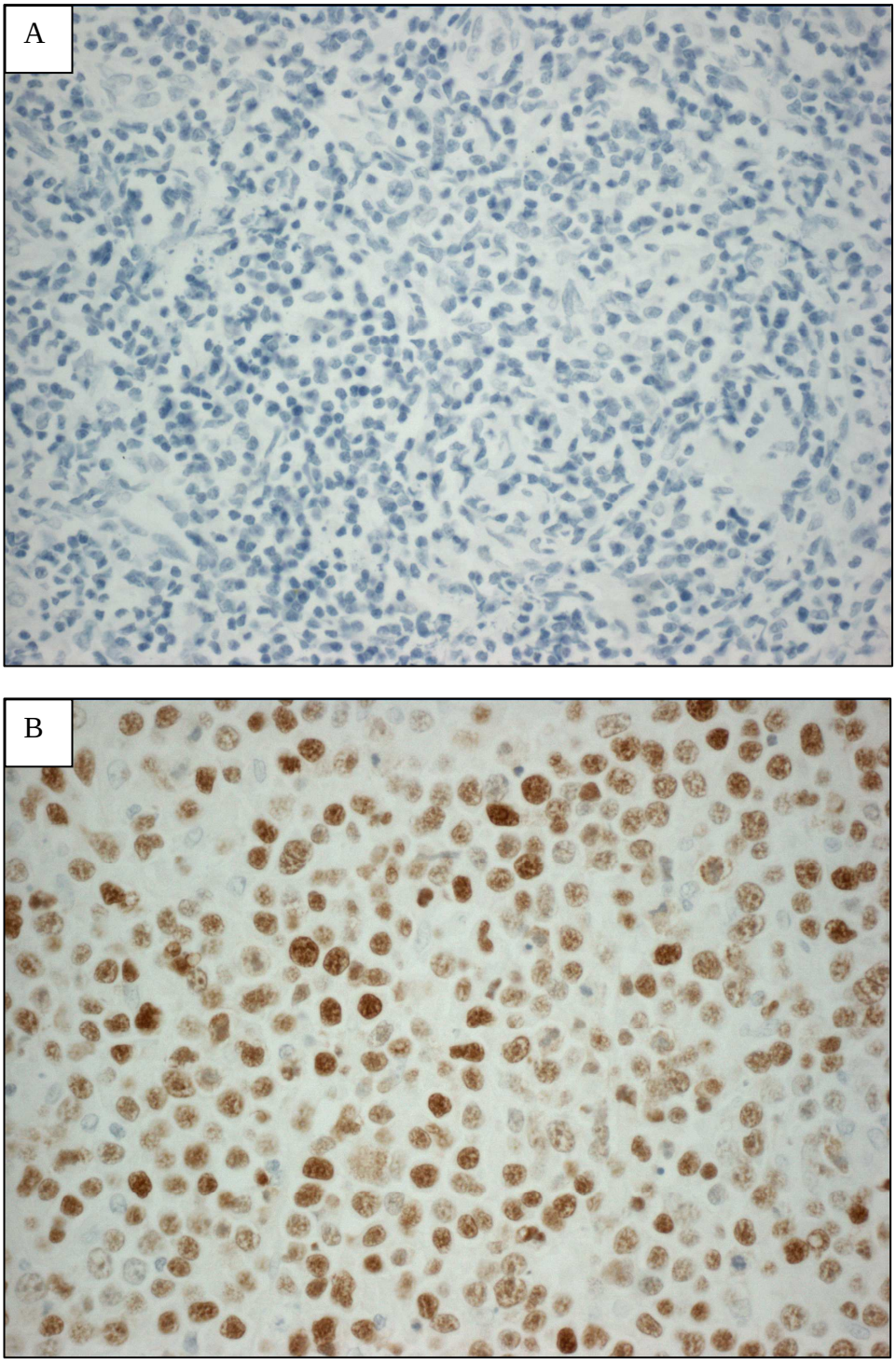
Figure 23 Western Blot of the BT549 and CRL2366 cell lines demonstrating a band at 21 Kda in both cell lines using the monoclonal antibody to p21 (monoclonal anti-human mouse anti-p21Cip1/WAF1 Clone EA10 (Zymed Laboratories, South San Francisco, California, USA))



3.3.4 Immunocytochemical Analysis of p53 Expression

Positive staining for p53 was assessed as more than 10% positive nuclear staining (Leroy et al. 2002). Seventy-seven blocks from 29 patients were sectioned, stained and analysed. Of these seventy seven samples, 14 had mutated *TP53* and 63 had wild type *TP53*. Immunocytochemistry was negative in 49 (64%) of these sections. Of the fourteen samples with known TP53 mutation, nine of the fourteen (64%) stained positively for p53 protein. There was a high association of immunocytochemical overexpression with the presence of missense mutations (82%). Sixty three samples were available from patients with wild type *TP53* and 45 of these cases (71%) showed negative staining. However, eighteen non-mutated samples stained positively for p53 protein (29%). (**Figure 24**)

Figure 24 p53 immunocytochemistry demonstrating, A) negative staining in FL OM x40, B) positive staining in DLBCL OM x40 from the same patient



3.3.5 Immunocytochemical Analysis of Mdm2 Expression

Nuclear staining was considered positive in the analysis of Mdm2 protein expression. Morphologically Mdm2 was seen to be expressed in the main by centroblasts and this was reflected in an increasing percentage of positive cells in higher grades of FL. Higher expression in DLBCL samples was seen (mean 72%; 95% confidence interval 68-76%) in comparison to FL (mean 58%; 95% confidence interval 54-62%). This observation was statistically significant $p < 0.001$. (**Figure 25 and Figure 26**)

Figure 25 Immunocytochemistry for MDM2, A) FL demonstrating some positive staining for MDM2 protein OM x40, B) DLBCL demonstrating increase in positive staining with MDM2 OM x40

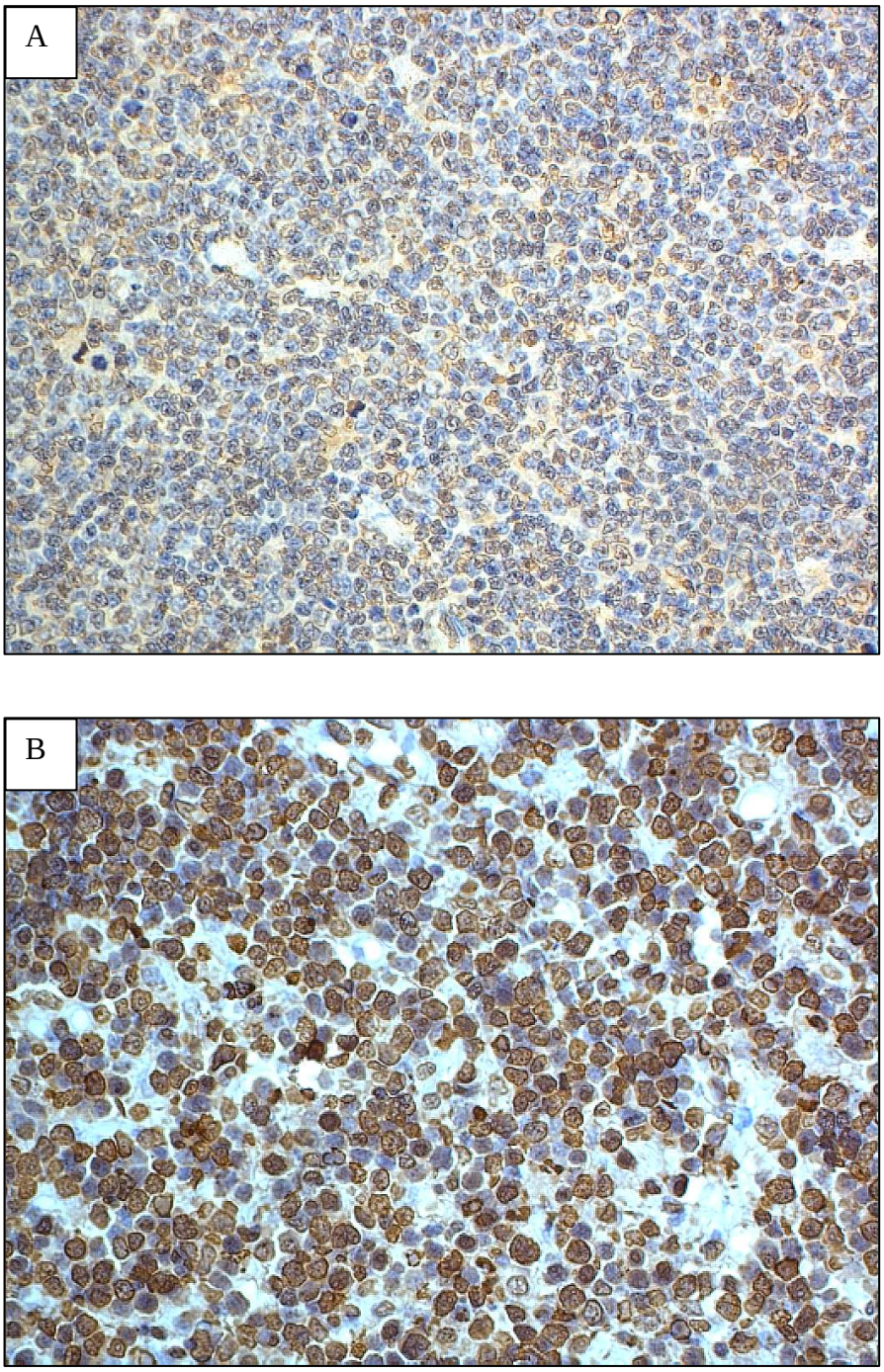
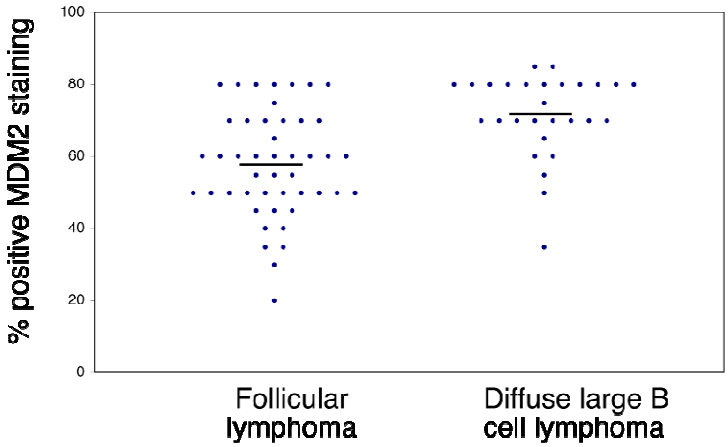


Figure 26 Comparison of MDM2 protein expression by immunocytochemistry in FL versus DLBCL. The mean level of expression is indicated by the black bar (Davies AJ et al. 2005 with permission).



3.3.6 Immunocytochemical Analysis of p21 Expression

Nuclear staining was considered positive in the analysis of p21 protein expression (Fredersdorf S et al. 1996). The majority of samples were negative for p21, with positive staining in nineteen of seventy-one samples assessed (27%). There was no correlation between p21 protein expression and histological type. There was no correlation between *TP53* mutation and p21 protein expression.

3.3.7 Correlation of p53, MDM2 and p21 Immunocytochemical Expression

There was no correlation between the expression of either MDM2 or p21 proteins and the presence of *TP*53 mutation. MDM2 expression was observed in 67% (95% CI: 63-70%) of samples with *wtTP53* and in 62% (95% CI: 55-68%) of samples with mutated *TP53*. Lack of p21 staining was present both in samples with *wtTP53* and mutated *TP53*.

3.4 Discussion

In contrast to other studies (Sander CA et al. 1993; Lo Coco F et al. 1993) which were performed on a smaller number of patients, this analysis of paired FL and transformed FL samples from a large cohort of patients' has shown that mutation of *TP*53 is not prerequisite for transformation in the majority of cases. Although no *TP53* mutations were present in initial diagnostic FL samples, the presence of *TP*53 mutation in FL samples prior to transformation (14% of samples) suggests that mutation may be an early event in the natural history of FL for some patients who eventually transform to high-grade lymphoma. The previous studies demonstrated widely disparate frequencies of *TP53* mutation with 26% (Sander CA et al. 1993) and 80% (Lo Coco et al. 1993) respectively in the transformed samples. The present study demonstrated mutations in recurrence samples in 28% of patients from the 91 biopsies analysed. In this study, the presence of a *TP53* mutation in FL did not correlate with reduced overall survival. This may be explained by data demonstrating that mutation of *TP*53 at codons 248 and 273 does not result in any alteration of protein conformation or abnormal localization. Indeed wild type activity could be elicited (Ory K et al. 1994). Codons 175, 248 and 273 are considered mutation hotspots accounting for 22% of mutations in human cancers (Caron de Fromental C, Soussi T 1992).

The dogma within the field of haematological malignancy, despite evidence from a study in 1993 (Villuendas R et al 1993), assumes the presence of *TP53* mutation based on positive immunocytochemical staining for p53 protein. This work has demonstrated that the presence of immunocytochemical staining cannot be used as a surrogate for genomic mutation detection in FL and transformed FL. This concurs with previous work which has shown over several histological types of non-Hodgkin's lymphoma that detection of protein over-expression is not dependent upon *TP53* gene mutation and conversely that the absence of protein does not exclude mutation. Nonsense mutations of TP53 will result in lack of detectable protein (Villuendas R et al. 1993). The detection of p53 protein by immunocytochemistry in the absence of *TP53* mutations has been previously observed in lymphomas (Martinez-Delgado B et al. 1997) and this observation indicates that there may be an alternative mechanism for accumulation of p53 protein independent of mutations. There has been increasing interest in the use of small

molecule inhibitors as therapeutic strategies in cancer particularly those which target the p53 pathway. It is essential that accurate determination of *TP53* mutational status is obtained if this therapy is to be used appropriately in the clinic as their therapeutic action depends the presence of wild type *TP*53 (Vassilev LT et al 2004).

It is evident from these results that transformation of FL to DLBCL is, in most patients, unrelated to *TP53* mutation. The increased expression of MDM2 in transformed DLBCL is independent of p53 status and suggests that it is the result of a different mechanism. Previous work in chronic lymphatic leukaemia (CLL) and other B cell NHL has produced similar results although over-expression of *MDM2* was seen at a higher frequency in lower grade lymphomas. There was a correlation with gene over-expression and advanced stage (Watanabe T et al. 1994). A separate study showed MDM2 over-expression in a small number of transformed DLBCL cases. Cases of indolent NHL demonstrated no MDM2 over-expression at diagnosis (Møller MB, Nielsen O, Pedersen NT 2002). Within FL a recent study has demonstrated an increased percentage of grades 3a and 3b FL to be MUM1-positive as compared to FL grades 1 and 2 (78.9% versus 7.4%) and 90% of the grade 3b FL cases were positive. In addition, there was a higher incidence of cases with a DLBCL component in MUM1-positive cases than MUM1-negative case (Naresh KN 2007).

There must be additional mechanisms driving the transformation process. Gene expression profiling has identified two gene expression signatures associated with transformation; one with increased expression of *c-myc* and genes regulated by *c-myc* and the second with decreased expression of these genes (Lossos IS et al 2002). Transgenic mice which overexpress the c-Myc oncogene under the control of the immunoglobulin heavy chain gene develop B cell lymphomas after a latent period of several months (Adams JM et al. 1985). Within this murine model *TP53* acts as a tumour suppressor and inactivation of the gene results in increased aggressiveness and speed of onset (Schmitt CA et al. 1999). Additionally, disruption of apoptosis by overexpression of BCL2 produces an advantage for tumour formation and is independent of the *TP53* mutational status (Schmitt CA et al. 2002). *Mdm2* haploinsufficiency in this model strikingly inhibits lymphoma development; transgenic mice with loss of one *Mdm2* allele had significantly delayed development of tumours and increased life span with increased susceptibility of B cell

lymphocytes to apoptosis. This suggests that targeting MDM2 may be an effective method of slowing or abrogating tumour development and may act by enhancing apoptosis via the p53 pathway (Alt JR et al. 2003). Recently, small-molecule inhibitors of MDM2, the Nutlins, have been developed. These compounds have been demonstrated to bind MDM2 in the p53-binding pocket. Cell lines treated with Nutlin-1 were shown to accumulate wild-type p53 and then elevate levels of MDM2 and p21 protein consistent with activation of the p53 pathway in these cancer cells. This increase in p53 protein was due to reduced degradation as transcription of the p53 gene was not affected by administration of the drug. Cell cycle arrest was demonstrated at the G1 and G2 phases. A variable dose dependent antiproliferative and cytotoxic effect was also observed which was linked to the p53 status of the cell lines. The IC_{50} values in cells with wild type p53 were considerably lower than in cells with mutant p53. Finally, Nutlin-3 can suppress the growth of murine tumour xenografts (Vassilev LT et al. 2004). Clinical trials of these drugs in patients with a variety of haematological malignancies have produced encouraging results. In acute myeloid leukaemia (AML), treatment with Nutlin-3 produced apoptosis in samples with wild-type p53. Inhibition of MDM2 enhanced the cytotoxic effects of doxorubicin and cytosine arabinoside in AML blasts (Kojima K et al. 2005). Similar results were demonstrated in myeloma, with apoptosis of neoplastic cells in the presence of bone marrow stromal cells, without damage to the stromal cells (Stühmer T et al. 2005). In chronic lymphatic leukaemia (CLL) Nutlin-3 produced apoptosis in cells with wild type p53. In addition, human T lymphocytes were less sensitive to nutlin-3 than CLL cells (Coll-Mulet L et al. 2006).

The results of this study establish mutations in *TP53* as being a minor contributor to the process of transformation however targeting MDM2 overexpression in transformed cases may be a more effective treatment option.

CHAPTER FOUR:

Validation of Tissue Microarray methodology for the evaluation of genes implicated in transformation

4.1 Introduction

The explosion of new technology such as cDNA microarrays has allowed the evaluation of gene expression in tissue samples. The results have revealed many potentially important genes involved in the development of malignancy in numerous types of tumours. This wealth of information has the potential to overwhelm scientists who are trying to identify those genes which are essential in the development of malignancy within a particular type of tissue. In order to determine if a gene has prognostic or therapeutic potential the protein expression in many samples from numerous patients needs to be analysed. Protein expression is more indicative of function than gene expression as numerous post-translational modifications may occur. There are limitations in assessment of protein expression. Traditional tissue samples are formalin fixed paraffin embedded tumour blocks. To assess protein expression in this material there must be an antibody to the protein of interest, which is effective in paraffin embedded tissue. In order that sufficient patient samples can be assessed to validate the importance of a particular gene numerous sections would need to be cut, stained and assessed. This is expensive in the use of valuable tissue samples, time and reagents.

4.1.1 Tissue Microarray

In order to address this disadvantage a technique called Tissue Microarray (TMA) has been developed. Small cores of tissue are taken from the paraffin embedded tissue blocks of the whole biopsy. To do this, haematoxylin and eosin slides of the whole tissue section are assessed and the areas of interest marked by the reviewing pathologist. The slide is aligned with the correct block and cores are taken from the area of interest and embedded into a fresh paraffin wax block. Core sizes can vary from 0.6mm to 3mm. The resulting block can contain as many as 1000 cores of tissue samples and depending upon the thickness of the donor blocks can allow as many as 150 sections to be cut from the same block. This has the result of amplifying rare material as well as providing a high throughput technique for assessment of protein expression. An additional advantage is that standardization of immunocytochemistry across all the tissue samples is achieved.

A concern with this technique is that the samples of tumour obtained will not be

representative of the biological properties of the entire tumour. Numerous studies have been carried out since the initial description of this technology, which indicate that with correct sampling this is not problematic. The data in the initial paper describing TMA showed a correlation of 84% between TMA immunocytochemistry and tissue homogenates for oestrogen receptor (ER) levels. This is in keeping with the correlation between full section immunocytochemistry and tissue homogenates for ER (Kononen J et al. 1998). A subsequent study in breast carcinomas showed that analysis of two 0.6mm tissue cores for the antigens of the oestrogen receptor (ER), progesterone receptor (PR) and Her2/neu was comparable to full sections in more than 95% of cases. The same authors also presented data suggesting that antigens within paraffin embedded tissues retain their antigenicity for many years, in some cases over six decades (Camp RL, Charette LA, Rimm DL 2000). A further study in breast malignancy assessed TMAs in the analysis of three prognostic markers in a series of 533 breast tumours. Four 0.6mm cores were sampled from each tumour, one core from the central area and three from peripheral regions. Evaluation of large section staining as compared to TMA was performed for ER, PR and p53. A single TMA core correlated with full sections in about 95% of cases for ER, 75-81% for PR and 70-74% for p53. However, a single core was sufficient for association between clinical outcome and molecular alterations to be identified (Torhorst J et al. 2001). Another study in a different disease entity assessed 59 fibroblastic tumours for their expression patterns of p53, ki-67 (a proliferation marker) and the product of the retinoblastoma gene (pRB) (Hoos A et al. 2001). The cut-off value for evaluating a case as positive was 10% for p53 and 20% for ki67. The assessment of pRB was more complex and included negative cases resulting from a lack of protein expression due to either mutation or deletion, strongly positive cases resulting from nonfunctional hyperphosphorylated protein (Cote RJ et al. 1998) and cases which were moderately positive as a result of expression of wild type protein. The results show that concordance with full section ICC is reduced in cases where complex phenotypes are assessed however an increase in the number of cores assessed, in this case to three, can improve this concordance. The analysis of three cores per sample resulted in concordance of 96%-98% for two phenotypes and 91% in assessing the more complicated pRB (Hoos A et al. 2001). Several studies have been performed using TMA in Hodgkins and Non-Hodgkins lymphomas with favourable correlation with full sections (Natkunam Y et al. 2001; Rassidakis GZ et al. 2002; Hedvat CV et

al. 2002).

4.1.2 Gene expression profiling in haematological malignancy

The value of gene expression profiling in haematological malignancies was demonstrated by early studies in *de novo* DLBCL which generated gene expression signatures capable of dividing patients into prognostic groups (Alizadeh et al. 2000). Further studies have focused on follicular lymphoma. The importance of the microenvironment in FL was highlighted by a study which analysed 191 lymph node biopsies and produced two signatures correlating with outcome. Immune response-1 (IR-1) was associated with good prognosis and included genes for T lymphocytes, immune response-2 (IR-2) correlated with poor prognosis and included genes for dendritic cells and some macrophage markers. These signatures were independent of clinical parameters (Dave S et al. 2004). A subsequent study analysed 80 FL patients lymph node biopsies and demonstrated that a number of genes could differentiate between aggressive and indolent disease and the use of these gene signatures could give a more accurate prediction of clinical behaviour than clinical parameters or histological grade (Glas AM et al. 2005).

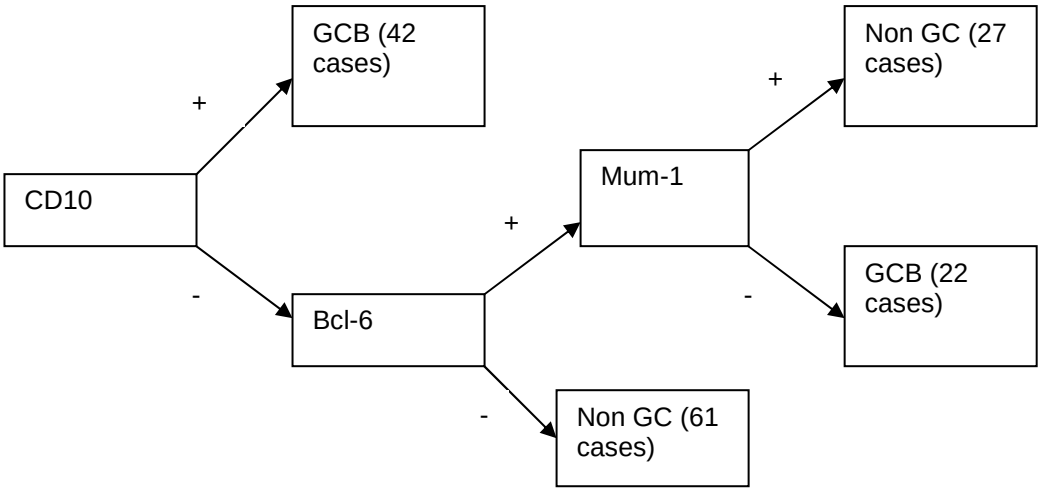
4.1.3 Activated-B-cell-like versus Germinal-centre-B-like groups of DLBCL

De novo DLBCL is a heterogeneous disease both morphologically and clinically. Durable remissions with anthracycline based chemotherapy are achieved in 40-50% of patients (Coiffier B 2001) however accurate prediction of patients who need aggressive treatment is essential. Currently the International Prognostic Index (IPI) is used for prognostication (The International Non-Hodgkin's Lymphoma Prognostic Factors Project 1993) however this has limited discriminative power. Gene expression profiling has allowed *de novo* DLBCL into prognostically significant subgroups; activated-B-cell-like (ABC), germinal-centre-B-like (GCB) and type 3. Patients with an ABC genotype have a worse prognosis (34% 5-year overall survival) than patients with GCB genotype (76% 5-year overall survival) (Alizadeh et al. 2000; Wright et al 2003). The type 3 group is heterogeneous but appears to have a similarly poor outcome to the ABC group. The use of cDNA microarrays is expensive and restricted to large laboratories, as well as requiring fresh tissue. The translation of these gene profiling experiments to immunocytochemistry would enable the use of this information in a much wider clinical setting as well as

validating the genetic data at a protein level on a large number of cases. Hans et al used immunoctyochemistry on TMA composed of 152 cases of DLBCL to evaluate the predictive markers CD10, BCL-6 and IRF4/MUM1 to accurately subdivide DLBCL into the prognostically relevant subgroups using cDNA microarrays as gold standard (Hans CP et al 2004).

GCB phenotype was indicated by CD10+, BCL-6-, MUM-1- or CD10-, BCL-6+, MUM-1- and non-GCB phenotype CD10-, BCL-6-, MUM-1+ or CD10-, BCL-6+/-, MUM-1- (**Figure 27**).

Figure 27 Decision tree for immunoperoxidase TMA classification of DLBCL (adapted from Hans CP et al. 2004)



The expression of CD10 or BCL-6 correlated with improved overall survival however expression of MUM1 was associated with worse outcome. Cases were classified using CD10, Bcl-6 and MUM1 expression and 64 cases (42%) classed as GCB and 88 cases (58%) non-GCB. The 5 year overall survival for the GCB group was 76% compared to 34% for the non-GCB group which was similar to the overall survival using the cDNA microarrays (Hans CP et al. 2004). This data demonstrated

that immunocytochemistry had a role in validating gene array data at a protein level in tumour tissue. This work presented here assessed the phenotype of DLBCL arising from transformed FL to ascertain whether the known germinal centre origin of FL is maintained on transformation.

4.1.4 Multiple Myeloma Oncogene 1/ Interferon Regulatory Factor - 4 (MUM1/IRF4)

The *MUM1* gene was first identified in multiple myeloma cells lines in which a recurrent translocation t(6;14)(p25;q32) positioned the immunoglobulin heavy-chain (*IgH*) locus next to *MUM1/IRF4* gene, a member of the interferon regulatory factor (IRF) family (Iida S et al. 1997). The resulting overexpression of *MUM1* gene has been shown to have a possible role in tumourigenesis (Iida S et al. 1997). Murine models deficient in IRF4 showed defective immunoglobulin production as well as defects in cytotoxic and anti-tumour T lymphocyte responses suggesting a functional role in both B and T lymphocytes (Mittrucker H et al. 1997). MUM1 has also been shown to alter the expression of cytokine genes in T lymphocytes. The production of interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 10 (IL-10) and interleukin 13 (IL-13) is strongly increased if MUM1 expression is produced in T lymphocytes which lack endogenous MUM1. MUM1 also interacts with a member of the NFAT family of transcriptional repressors and immunosuppressants which target NFAT activation can abrogate the MUM1 induced production of cytokines (Hu CM et al. 2002). Stable expression of MUM1 in a lymphoid cell line in which it is normally deficient produces an enhanced apoptotic response on engagement of the Fas receptor. Mice deficient in MUM1 have defective activation-induced cell death suggesting that MUM1 is important in the regulation of apoptosis within lymphoid cells (Fanzo JC et al. 2003). Subsequent development of a monoclonal antibody Mum1p demonstrated tissue expression of the human MUM1/IRF4 protein in the cytoplasm and nuclei of plasma cells and in a small number of light zone germinal centre B lymphocytes. The positive germinal centre B lymphocytes differed phenotypically from the majority of germinal centre B lymphocytes and mantle B lymphocytes. The MUM1 positive cells were Bcl6 and Ki67 negative whereas MUM1 negative cells were Bcl6 and Ki67 positive and mantle cells were negative for all three markers. Analysis of single MUM1 positive germinal centre cells using polymerase chain reaction confirmed B cell origin and the number of somatic

mutations demonstrated the cells to be of germinal centre or post germinal centre stage. In addition, there was expression of the MUM1 protein in lymphoplasmacytoid lymphomas, multiple myeloma and three quarters of diffuse large B cell lymphomas (DLBCL). There was coexpression of Bcl-6 and MUM1 in approximately half of the DLBCL whereas the expression of these markers in normal B lymphocytes is mutually exclusive (Falini B et al. 2000).

4.1.5 Forkhead box protein P1 (Foxp1)

Foxp1 is a member of the Fox family of winged helix/forkhead DNA-binding proteins which act as transcriptional repressors. Foxp1 maps to the chromosome region 3p14.1 and many tumours show alterations in this region (Pandis N et al. 1993; Fujino T et al. 1994). Analysis of both mRNA and protein expression levels has demonstrated a difference between normal and malignant tissue. Immunohistochemical analysis of a variety of malignant tumours demonstrated altered levels and location of expression of the protein in many neoplastic cells. Within normal tissues, the presence of FOXP1 protein has been observed in B lymphocytes in the mantle zone and some germinal centre cells (Banham AH et al. 2001). Absence of the *Foxp1* gene causes a defect in the early development of B lymphocytes. There is a block on transition from pro-B cell to pre-B cell and reduced levels of V(D)J recombination (Hu H et al. 2006). Analysis of diagnostic lymph node biopsies from patients with DLBCL using immunohistochemistry has demonstrated a high level of expression in 23/126 patients. The presence of FOXP1 correlated with poor prognosis and was only observed in patients who lacked the GC phenotype (Barrans SL et al. 2004). Further investigation has revealed a significant association between the expression of FOXP1 and worse survival in patients with DLBCL. Patients with FOXP1 positive tumours had a median overall survival of 1.6 years in comparison to patients with FOXP1 negative tumours who had a median overall survival of 12.2 years (Banham AH et al. 2005). Follicular lymphoma cells demonstrated positive expression of Foxp1, by immunohistochemistry in 11 out of 13 cases, with all being of lower grade. The two cases which did not express FOXP1 were grade 2 or 3 (Brown P et al. 2005). The expression of FOXP1 has also been shown to correlate with outcome in patients with de novo MALT lymphomas and indeed identify a subset of these low grade lymphomas which were likely to transform to aggressive DLBCL (Sagaert et al 2006). As part of the study

investigating antigenic differences between ABC and GCB DLBCL Hans et al also evaluated other immunohistochemical markers including FOXP1. Positive staining for FOXP1 was observed in 61% of DLBCL and its expression was seen in 71% of non-GCB cases as compared to 48% of GCB cases. There was no correlation with overall survival or event free survival (Hans CP et al. 2004). In addition to identifying whether FL transformed to DLBCL of GC or ABC like phenotype we wished to see if the FOXP1 result in de novo DLBCL was replicated in transformed FL.

4.1.6 Gene expression profiling of pre- and post-transformation sample of FL

Previous gene expression profiling on haematological malignancies suggested that gene expression profiling is a powerful tool with which to examine potential genetic differences in pre- and post transformation samples.

As previously indicated, transformation of follicular lymphoma to an aggressive high grade lymphoma is a common and adverse prognostic event. The gene expression data was obtained from lymph node samples of follicular lymphoma (FL) which subsequently transformed to diffuse large B cell lymphoma (DLBCL) using the Lymphochip cDNA microarray. A list of seventy-six genes whose expression was significantly altered on transformation was generated using the random variance model *t*-test. The cut off value for *P* was set at <0.001. Twenty nine genes demonstrated up-regulation and 47 genes are down-regulated upon transformation (Davies AJ et al. 2006). In order to assess concordance between mRNA and protein expression, gene products were chosen for further study using immunocytochemistry. Within the up-regulated genes *nucleoside dephosphate kinase A (nm23-H1)* which showed a $\log_2$ fold change of 0.85 was chosen for further study. This gene was of additional interest as elevated serum levels of nm23-H1 have been detected by ELISA in patients with aggressive non-Hodgkin's lymphoma and acute myeloid leukaemia and correlated with poor prognosis (Niitsu N et al 1999; Niitsu N et al 2000). The fold change represents the average change in expression ($\log_2$) across 20 samples between FL and DLBCL. One of the up-regulated genes with the power to differentiate between FL and transformed DLBCL samples is a chromosomal passenger protein called *Aurora Kinase B (AURKB)*. Although less significantly up-regulated than the other genes, with a $\log_2$ fold change of 0.64,

AURKB was worthy of investigation because a small molecule inhibitor of *AURKB* was already being investigated in acute myeloid leukaemia. *CR2 (CD21)* and *FCER2 (CD23)* were the most significantly down-regulated and their protein expression was analysed. The expression of a third protein, FOXP1, was also analysed. Although this gene was not differentially expressed between FL and transformed FL, the presence of FOXP1 correlated with poor prognosis in *de novo* DLBCL and was only observed in patients who lacked the GC phenotype (Barran SL et al. 2004). As part of this study was to investigate the phenotype of transformed FL we wished to investigate whether the FOXP1 result in *de novo* DLBCL was replicated in transformed DLBCL. The protein expression of these genes was analysed on an independent validation set of 34 patients with at least one pre-transformation and one post-transformation paraffin embedded biopsy.

4.1.7 Aurora Kinase B (AURKB)

Cellular division is an essential and complex process and, of necessity, is tightly regulated. The final stage of mitosis is cytokinesis where two daughter cells are produced each containing a single complete set of chromosomes. In order to achieve this, during the cell cycle chromosomes must be correctly bi-orientated and aligned. Extensive investigation into the mechanisms by which this occurs have been undertaken. Within Drosophila a serine/threonine protein kinase, Aurora, was identified where mothers with mutant *aurora* would produce embryos with closely paired centrosomes at inappropriate stages in mitosis. In addition these embryos would develop interconnected spindles with shared poles. In larvae with amorphic alleles there is mitotic arrest and pupal lethality with the formation of large circular monopolar spindles suggesting that the centrosomes have failed to separate and form a bipolar spindle (Glover DM et al. 1995). The budding yeast *Saccharomyces cerevisiae* possesses a kinase Ip1l which is required to ensure chromosome segregation. In yeast with mutant kinase uneven DNA staining density was present near the ends of opposite elongated spindles suggesting unequal segregation of chromosomes at the poles (Franscisco L, Wang W, Chan CSM 1994). A mammalian version related to *Drosophila* Aurora and *Saccharomyces cerevisiae* Ip1l was identified in the rat. This was named Aurora and Ip1l-like midbody-associated protein (AIM-1). Both mRNA and protein for AIM-1 are expressed in the G2/M phase of the cell cycle and begin to localize as a broad band in the midzone of the

central spindle in late anaphase. By telophase and cytokinesis AIM-1 is concentrated at the midbody. The expression of kinase inactive AIM-1 often results in failure of cytokinesis with resulting cell polyploidy (Terada Y et al. 1998). In humans, two homologues of the *Drosophila* Aurora and yeast Ip1l kinase genes were initially identified and named *aurora1(aurora B)* and *aurora2 (aurora A).* They were identified using a PCR screening technique aiming to identify protein kinases which were overexpressed in colonic carcinomas. The aurora2 kinase was demonstrated to be equivalent structurally and functionally to the yeast kinase Ip1l whereas aurora1 kinase demonstrated a different biological activity. Both aurora1 and aurora2 mRNA and protein levels and activity were highest at the G2/M phase of the cell cycle although aurora2 kinase activity was maximal prior to the highest level of aurora1 activation. Both kinases were demonstrated to localize to mitotic structures suggesting a role in chromosome segregation. The *aurora2* gene was shown to map to chromosome 20q13 (Bischoff JR et al. 1998). This locus is overexpressed in many malignancies (Muleris M et al. 1987, Bigner SH et al 1988, Iwabuchi H et al 1995, Kallioniemi A et al. 1994, Tanner MM et al 1994, Tanner MM et al 1996). Aurora2 mRNA levels were demonstrated to be up-regulated in numerous tumour cell lines. In addition, Aurora2 DNA was amplified and its RNA overexpressed in 52% of primary rectal carcinomas studied. Overexpression of *aurora2* is able to transform rat fibroblasts suggesting its role as an oncogene. High expression of *aurora1* transcript is seen in tissues with a rapid turnover such as fetal liver and epithelial cells but low levels are present in normal tissues. It is also overexpressed in many diverse tumour cell lines (Bischoff JR et al. 1998). These findings taken with the observations by Terada et al. that expression of AIM1 mutant disrupts the formation of the cleavage furrow and prevents separation at the final stage of cytokinesis suggest that members of the aurora1 kinase family are required for the completion of cytokinesis. A defect in this family would result in cells with abnormal amounts of DNA and polyploidy, findings which are often seen in malignant cells. Aurora B (Ip1lp) phosphorylates kinetochore components in budding yeast and is essential for ensuring chromosome segregation by correcting errors in microtubule attachment (Biggins S et al 1999). In order to achieve this Aurora B kinase targets checkpoint proteins BubR1, Mad2 and Cenp-3 to kinetochores resulting in the coupling of chromosome alignment with anaphase (Ditchfield C et al 2003). Aurora kinase B also phosphorylates the microtubule depolymerase MCAK and regulates both its

location and activity during mitosis suggesting that Aurora kinase B may biorient chromosomes by directing MCAK to depolymerise incorrectly oriented kinetochore microtubules (Lan W et al 2004). The addition of an Aurora kinase B inhibitor, Hesperadin, to mammalian cells increased the incidence of mal-orientated chromosomes (Hauf S et al 2003). The small molecule inhibitors ZM447439 and VX-680 inhibit both the proliferation and survival of tumour cells (Ditchfield C et al 2003; Harrington EA et al 2004). However, ZM447439 is not selective for Aurora B and also inhibits Aurora kinase A (Ditchfield C et al 2003) and VX-680 inhibits Aurora A more potently than Aurora B (Harrington EA et al 2004). Recent work had demonstrated that inhibition of Aurora B kinase activity produces similar effects to those achieved by ZM447439 and a novel compound which is over 100 times more specific for Aurora kinase B over Aurora kinase A in vitro produces similar phenotypes to those produced by ZM447439. Inhibition of Aurora kinase B activity produces a profound anti-proliferative effect indicating that it is an attractive drug target (Girdler F et al 2006).

4.1.8 Nucleoside dephosphate kinase A (nm23)

The nm23 gene family is a family of metastasis suppressor genes important in oncogenesis and metastasis. The first member of the family identified was nm23-H1 and its levels were reduced in highly metastatic tumours in rodents in comparison to tumours with low metastatic ability. The mechanisms by which they mediate these effects are unclear (Steeg PS et al. 1988; Lascu I, Gonin P 2000). Nm23-H1 is a nucleoside diphosphate kinase (NDP kinase) (Wallet V et al. 1990; Biggs J et al 1990) which acts to provide cells with high energy nucleosides other than ATP (Parks RE Jr, Agarwal RP 1973). Elevated serum levels of nm23-H1 have been detected by ELISA in patients with aggressive non-Hodgkin's lymphoma and acute myeloid leukaemia and correlated with poor prognosis (Niitsu N et al 1999; Niitsu N et al 2000). In addition, serum nm23-H1 levels have been shown to be an independent prognostic factor in patients with DLBCL and peripheral T cell lymphoma (Niitsu N et al. 2001). Analysis of tissue DLBCL samples using immunocytochemistry demonstrated strong positivity in 58.1% and this correlated with the serum level. The overall survival and progression free survival rates were lower in patients with tumours positive for nm23 in comparison to patients with nm23 negative tumours. This difference was also identified on comparison of the

serum levels of nm23 suggesting that the lymphoma cells produce nm23 (Niitsu N et al 2004).

4.1.9 Complement Receptor 2 (CR2/CD21) and CD23/FcεRII

Follicular dendritic cells are essential to both normal and neoplastic follicle formation within lymphoid tissue and are essential for adhesion and proliferation of B lymphocytes (Kosco MH, Pflugfelder E, Gray D 1992). The antibodies for CD21 and CD23 are used routinely to demonstrate the presence of FDC meshwork. Several studies have suggested that loss of FDC antigen in the stroma of FL may serve as a surrogate marker of tumour progression (Chang KC et al 2003). An immunocytochemical study observed the presence of FDC meshworks as more clearly defined in low grade lymphomas in comparison to high grade and loss of the FDC meshworks prior to transformation despite morphologically clear follicles being present (Shiozawa E et al 2003). The gene expression profiles demonstrated the most significantly down-regulated genes on transformation of FL to DLBCL were *CR2/CD21* and *CD23* and so their protein levels were analysed in tissue using immunocytochemistry.

4.1.10 Aims

The aim of this work was initially to validate TMA as a method to assess protein expression by innumocytochemistry in FL and DLBCL. Once the methodology was validated, a further study to examine the results of TMA use within our department as compared to published results was performed. Finally, using an independent set of pre-transformation and post-transformation patient samples to those analysed in the gene expression set (Davies AJ et al. 2006) TMA was used to validate the results obtained by gene expression profiling at a protein level and try to gain an insight into the mechanisms of transformation as well as confirm novel up-regulated genes as potential prognostic biomarkers or therapeutic targets.

4.2 Materials and methods

4.2.1 Patient selection

Ethics approval was obtained from the local regional ethics committee. Paired pre and post transformation samples (n=126) were obtained from 35 patients diagnosed with FL who subsequently transformed to DLBCL. A second TMA comprising 31 *de novo* DLBCL samples was produced for methodological validation.

4.2.2 Construction of Tissue Microarray

Tissue microarrays (TMA) were constructed of 1mm cores of patient tissue taken from representative areas of lymphoma (>5 neoplastic follicles for FL) using pre-marked H&E stained sections and from reactive tonsil and appendix controls, in triplicate using a manual arrayer (Beecher Scientific, Silver Spring, MD) as previously described (Hedvat CV et al. 2002; Kononen J et al. 1998). Approximately 120 cores were applied per slide. All cores from a single patient were randomly included on the same TMA.

4.2.3 Immunohistochemistry of validation panel

Paraffin embedded 4μm sections were cut and placed on TESPA coated slides, dewaxed and blocked with hydrogen peroxide/methanol solution. Antigen retrieval was performed as detailed in Table 4. Primary antibody was diluted as detailed in **Table 4**. Sections were then stained using the Vector Elite ABC kit (PK6100, Vector Laboratories, Peterborough, UK) and chromogen diaminobenzidine (Biostat, Stockport, UK).

Table 4 Validation panel antibodies

ANTIBODY	MANUFACTURER AND CLONE	CONTROL	ANTIGEN RETRIEVAL	DILUTION
CD3	Labvision CD-3-SP7	Tonsil	Pressure cooking	20:5000
CD5	Novocastra NCL-CD4-368	Tonsil	Pressure cooking	1:50
CD10	Novocastra NCL-CD10-270 clone 56C6	Tonsil	Pressure cooking	25:1000
CD20	Dako M755	Tonsil	Pressure cooking	20:8000
CD21	Novocastra NCL-CD21-2G9	Tonsil	Pressure cooking	1:50
CD23	Novocastra NCL-CD23-1B12	Tonsil	Pressure cooking	1:50
BCL2	Dako clone 124	Tonsil	Pressure cooking	100:5000
BCL6	Dako clone PG-B6p	Tonsil	Pressure cooking	100:4000
Ki67	Dako M7240	Tonsil	Pressure cooking	10:10000

4.2.4 Optimisation of novel antibodies for immunohistochemistry

Paraffin embedded 4μm sections were cut and placed on TESPA coated slides, dewaxed and blocked with hydrogen peroxide/methanol solution. Data sheets provided by antibody manufacturers provided appropriate control for titration and detail antigen retrieval. If no pretreatment was advised three different techniques were tried; no pre-treatment, pronase digestion for 15 minutes and pressure cooking. The primary antibody dilution was applied in dilution 1/50, 1/100 and 1/200 unless data sheet advised otherwise. The primary antibody was diluted with 1% BSA and azide. Sections were then stained using the Vector Elite ABC kit (PK6100, Vector Laboratories, Peterborough, UK) and chromogen diaminobenzidine (Biostat, Stockport, UK). Staining specificity and intensity was assessed and dilution further titrated if necessary. If slides were staining correctly with a known positive control all details of manufacturer, clone, pretreatment requirements, antibody dilution and known positive control were recorded for future use.

4.2.5 Immunohistochemistry of novel antibodies

This was carried out as described in the above sections. Antibody retrieval and the dilutions used are presented in **Table 5**.

Table 5 Novel antibodies dilution and antigen retrieval

ANTIBODY	MANUFACTURER AND CLONE	CONTROL	ANTIBODY RETRIEVAL	DILUTION
Aurora B	Abcam (ab2254)	Cervix	Pressure cooking	1:600
Nm23	Novocastra (NCL-nm23 clone 37.6)	Tonsil	None required	1:250
FoxP1	Gift from A. Banham, Oxford	Tonsil	Pressure cooking with tyramide amplification	1:40
Mum1/IRF4 (Multiple Myeloma Oncogene 1)	Dako (MUM1p)	Tonsil	Pressure cooking	1:100

4.2.6 Analysis of Tissue Microarrays

The entire TMA cores were analysed by myself and independently by another histopathologist and the number of positive cells expressed as an average percentage per high power field (x40 magnification). In the case of small differences in percentage (5-10%) an average of the two scores was used as the score. In the cases of large differences of score between the two histopathologists (<10% of cases) the cases were reviewed by both the scoring histopathologists and consensus reached in all cases.

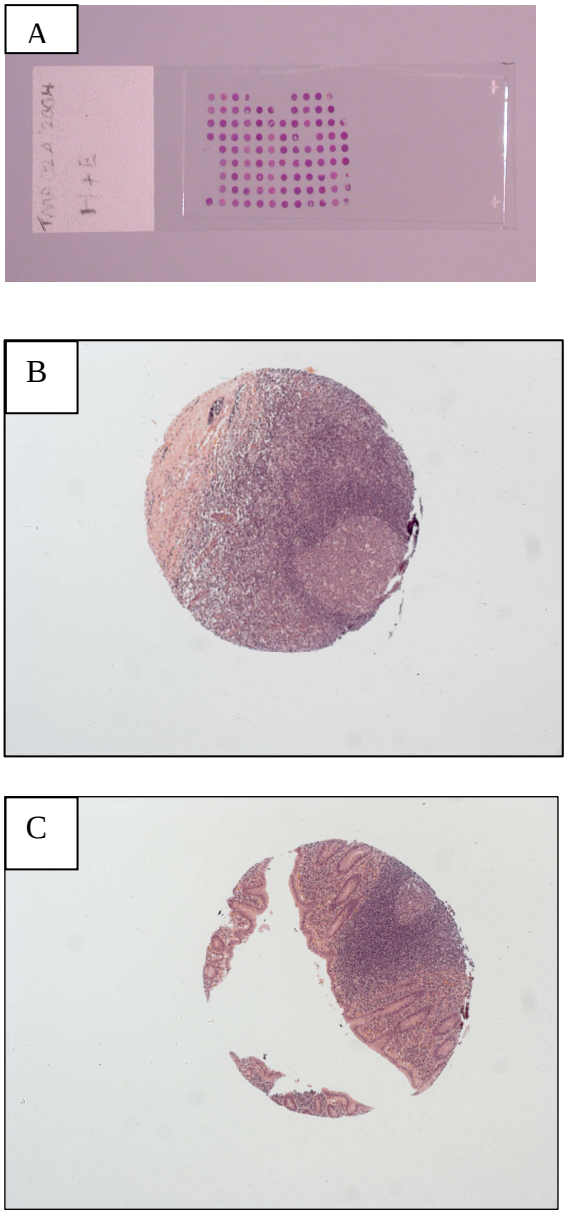
4.3 Results

4.3.1 Establishment of Transformed Tissue Microarray (TMA)

To accommodate the samples, four TMAs (transformation TMA) were produced each containing approximately 96 cores (**Figure 28A**). Each patient sample was arrayed in triplicate and control lymphoid tissue cores (appendix and tonsil) were included in each array (**Figure 28B and C**). Complete pre and post transformation sample pairs were assessable in 100 samples (35 patients).

In addition, a separate array composed of de novo DLBCL was constructed containing 31 initial diagnostic samples of tumour from 31 patients arrayed in triplicate. Control lymphoid tissue cores (appendix and tonsil) were included in the array.

Figure 28 Tissue Microarray composition, A) H&E slide of TMA, B) H&E stained reactive tonsil core 1 mm diameter OMx5, C) H&E stained appendix core 1 mm diameter OMx5



4.3.2 Correlation of TMA cores with full sections

To evaluate the accuracy with which the TMA cores were representative of full tissue sections scoring of routine diagnostic immunocytochemistry (CD3, CD5, CD10, CD20, CD21, CD23, BCL-2, BCL-6 and Ki67) was performed on TMA cores and full sections of ten samples identified at random from the cohort of patients in the study. The concordance between TMA and full sections was >90% (**Table 6**).

Table 6 Correlation of TMA cores with full sections immunocytochemistry

Antibody assessed	**TMA scoring correlated with full section scoring**	**TMA scoring did not correlate with full section scoring**
CD3	100% (10/10)	0% (0/10)
CD5	100% (10/10)	0% (0/10)
CD10	100% (10/10)	0% (0/10)
CD20	100% (10/10)	0% (0/10)
CD21	100% (10/10)	0% (0/10)
CD23	50% (5/10)	50% (5/10)
Bcl-2	100% (10/10)	0% (0/10)
Bcl-6	90% (9/10)	10% (1/10))
Ki-67	80% (8/10)	30% (3/10)
Overall correlation = 91%		

Corroboration of gene expression profiling has previously been performed using immunocytochemistry and TMA by Hans et al. (2004) who demonstrated reproduction of gene expression results by immunocytochemistry in 71% of GCB and 88% of non-GCB cases as compared to cDNA microarray (Hans CP et al. 2004). This result was used as a yardstick for this work.

4.3.3 Transformed FL is mainly of germinal centre (GC) like phenotype

The antibody panel identified by Hans et al. (2004) was applied initially to a TMA composed of 31 *de novo* DLBCL samples arrayed in triplicate to ensure that it performed as published. The adequacy of this TMA was assessed using a routine panel of diagnostic antibodies (CD3, CD5, CD10, CD20, CD21, CD23, CD138,

BCL-2, BCL-6, Ki67). If only two cores from a sample were assessable the consensus of two cores was accepted (5% of cases). In the event of the loss of two cores with only a single assessable core, the result from this was disregarded and marked as absent.

In the *de novo* DLBCL TMA 11/31 (35%) had a GC phenotype. Of these 11 cases, 6 cases (55%) were CD10+ and 5 cases (45%) were CD10-, BCL-6+, MUM-1-. This was similar to the results obtained by Hans et al. (2004) who observed 42% of 152 DLBCL cases analysed were GC type. The 20/31 non GC cases (65%) were composed of 18/20 (90%) CD10-, BCL-6+, MUM-1+ and 2/20 (10%) CD10-, BCL-6-. The 5 year overall survival for GC patients in this study was 73% and 51% for non-GC patients (p=0.00002). The 5 year overall survival for GC patients in the study by Hans et al. (2004) was 76% compared to 34% for the non-GC patients.

The transformation TMA was then used to investigate the phenotype of transformed DLBCL. Of the transformation samples, 31/35 (89%) demonstrated a GC-like phenotype (28/35 (80%) CD10+ and 3/35 (9%) CD10-, BCL-6+, MUM-1-). The remaining cases, 3/35 (9%) were of non-GC phenotype (CD10-, BCL-6+, MUM-1+) and one case was unclassifiable due to an absence of scorable cores for MUM-1.

4.3.4 Identification of proteins of potential therapeutic value

Of the genes which were significantly up-regulated on transformation in the previously described gene array data AURKB and nm23 were chosen for further study.

Levels of AURKB transcript were demonstrated to increase on transformation in a subset of patients. Analysis of the AURKB protein by immunohistochemistry demonstrated a nuclear staining pattern with obvious staining of chromosomes undergoing mitosis, in keeping with its role as a chromosomal passenger protein. Within normal germinal centres very few centroblasts (<5%) were positive for AURKB and this value was taken as negative for the neoplastic samples. AURKB protein expression increased on transformation in 13/33 (40%) of patients (**Figure 29**). There was low expression of the protein in both FL and transformed DLBCL in 18/33 (55%) of patients and a decrease in protein level in 2/33 (6%) of patients.

Duration of survival was not significantly different from transformation in patients with increased AURKB on transformation compared to those with decreased expression (p=0.6) (**Figure 30**). There was no difference in overall survival either in patients with increased or decreased expression of AURKB on transformation (**Figure 31**).

Figure 29 Immunocytochemistry for AURKB demonstrating increase in expression in DLBCL transformed from FL, A) FL OM x40, B) DLBCL OM x40

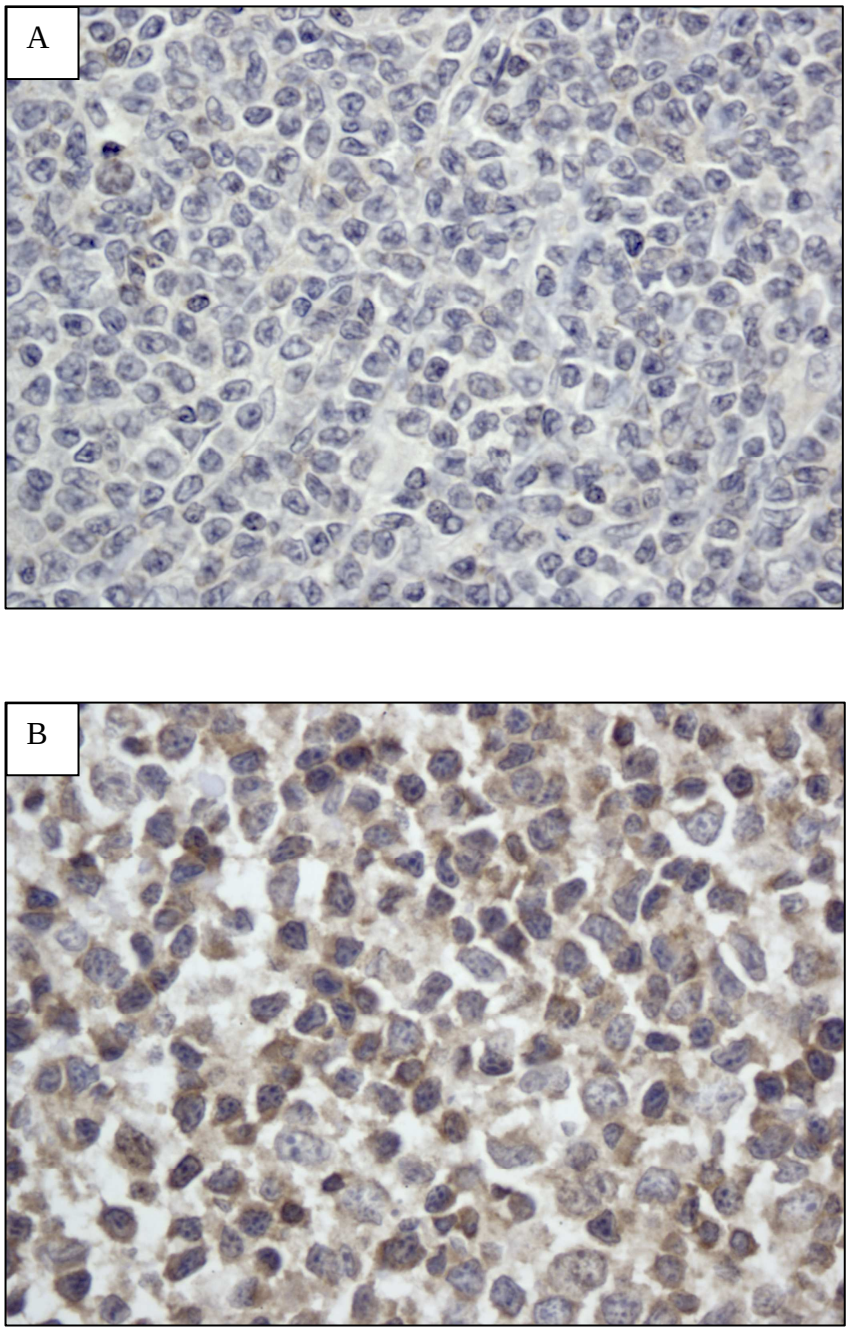


Figure 30 Kaplan-Meier estimate of survival from transformation by Aurora Kinase B expression (p=0.8).Blue indicates samples which were negative for expression of Aurora Kinase in both the FL and transformed DLBCL samples. Red indicates those samples where an increase in expression of Aurora kinase was observed in the transformed DLBCL sample.

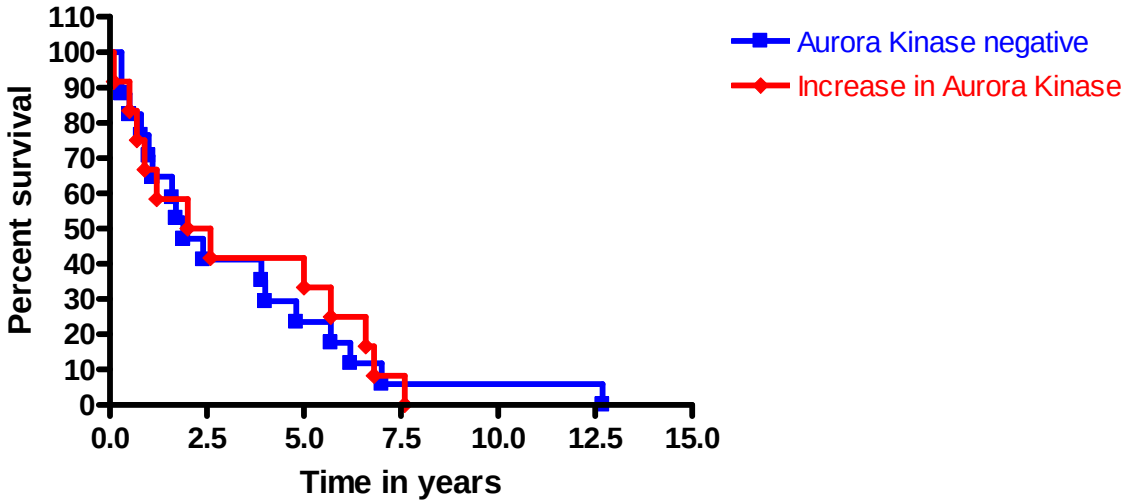
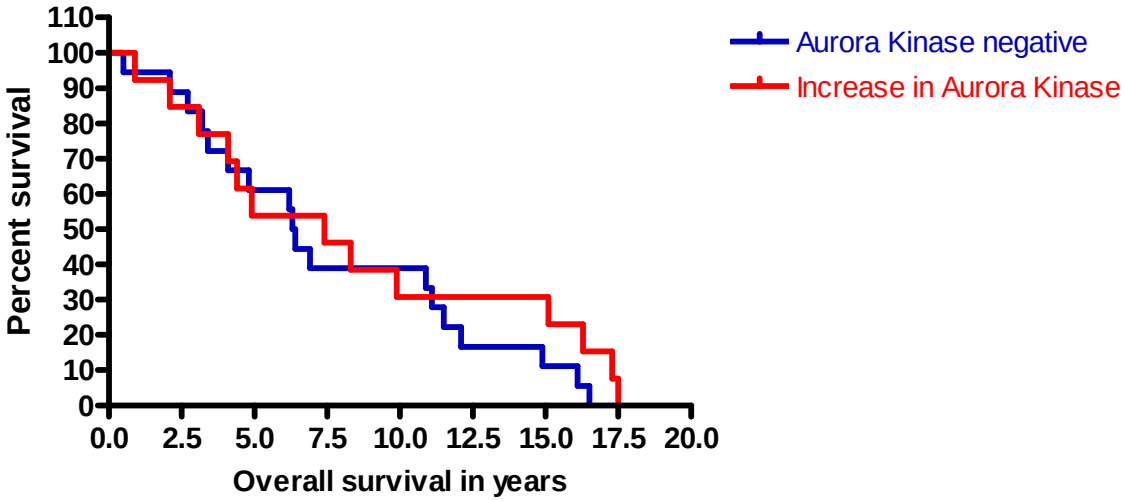
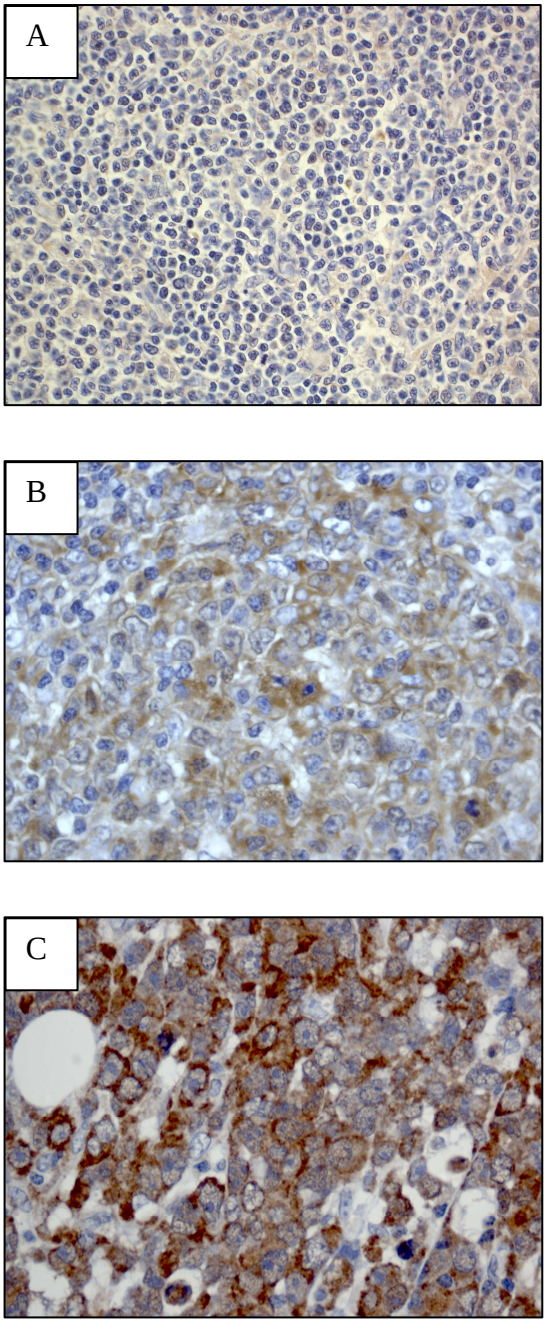


Figure 31 Kaplan-Meier estimates of overall survival of patients with transformed FL by expression of AURKB (p=0.2). Blue indicates samples which were negative for expression of Aurora Kinase in both the FL and transformed DLBCL samples. Red indicates those samples where an increase in expression of Aurora kinase was observed in the transformed DLBCL sample.



Analysis of nm23 protein by immunocytochemistry demonstrated weak positive cytoplasmic staining in endothelial cells in both FL and DLBCL in accordance with previous work (Niitsu N et al 2004). Only cytoplasmic staining within tumour cells was considered to be positive (**Figure 32**).

Figure 32 Immunocytochemistry staining for nm23, A) FL demonstrating absence of staining for nm23 OM x20, B) FL staining positively for nm23 OM x40, C) DLBCL staining positively for nm23 OM x40



Expression of nm23 protein increased or remained high on transformation in 12/23 (52%) of patients and decreased or remained low in 11/23 (48%). There was no difference in overall survival of patients with increased or decreased expression of nm23 or in time to transformation (**Figures 33 and 34**).

Figure 33 Kaplan-Meier estimates of overall survival of patients with FL who transform to DLBCL based on expression of nm23 (p=0.43). Blue indicates cases with low expression of nm23 in both the FL and transformed DLBCL or a decrease in expression on transformation of FL to DLBCL. Red indicates cases with high level of expression of nm23 in both FL and transformed DLBCL or an increase in expression level on transformation.

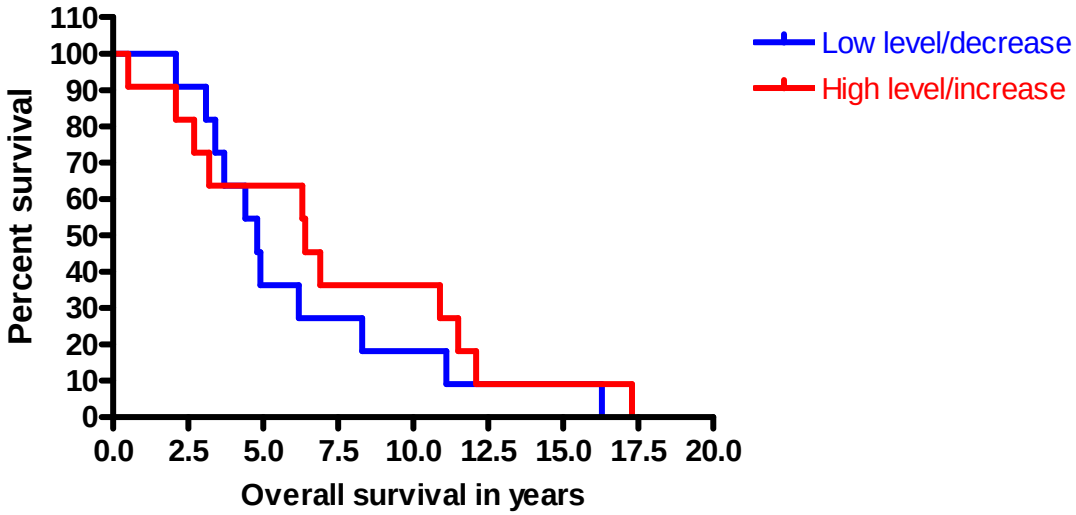
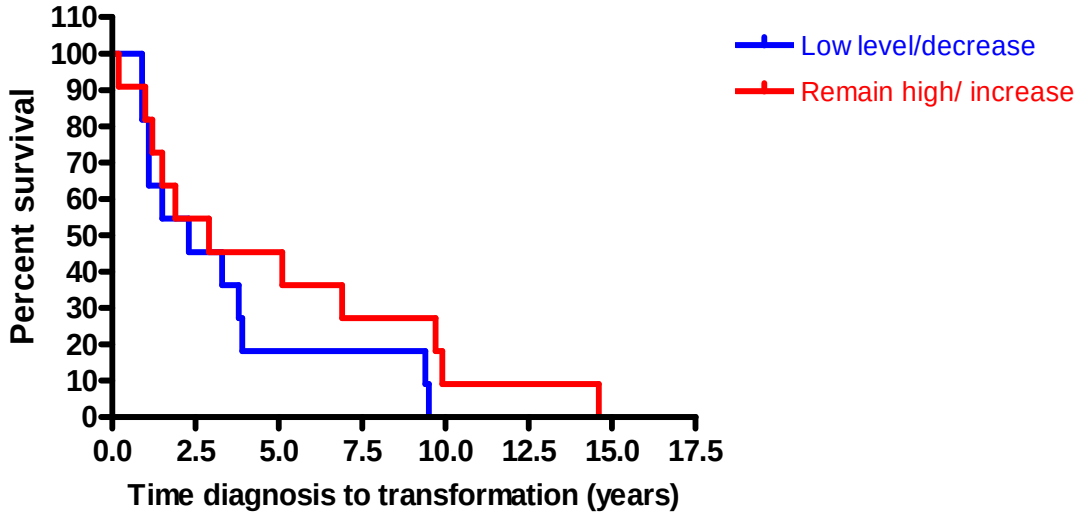


Figure 34 Kaplan-Meier estimates of time to transformation of patients with FL who transform to DLBCL based on expression of nm23 (p=0.22). Blue indicates cases with low expression of nm23 in both the FL and transformed DLBCL or a decrease in expression on transformation of FL to DLBCL. Red indicates cases with high level of expression of nm23 in both FL and transformed DLBCL or an increase in expression level on transformation.



4.3.5 Analysis of FOXP1 expression in *de novo* DLBCL and transformed FL

Assessment of staining with the antibody to FOXP1 demonstrated clean nuclear staining and the cutoff for positive staining was set at 30% in keeping with other studies (Barrans SL et al. 2004; Banham AH et al. 2005). Of the 31 cases of *de novo* DLBCL analysed, 11/31 (35%) were classified a GC phenotype and 20/31 (65%) were of non-GC phenotype. Thirty cases were available for analysis of FOXP1 expression, 22/30 (73%) staining positively and 8/30 (27%) showing staining at levels less than 30% (classed as negative). Of the GC phenotype cases (n=11); 6 cases (60%) were FOXP1 positive and 4 cases (40%) were FOXP1 negative. One case was unassessable for FOXP1 staining due to insufficient cores available for assessment. The non-GC phenotype cases (n=20) demonstrated positive staining in 16/20 (80%) and negative staining in 4/20 (20%).

Within the t-FL group all three cases of non-GC phenotype demonstrated positive staining of tumour cells (100%). These results are similar to those observed by Hans et al. (2004) who demonstrated increased expression of FOXP1 in non-GC phenotype cases (Hans CP et al. 2004).

4.4 Discussion

In common with previous other studies (Kononen J et al. 1998; Hoos A et al. 2001), this work demonstrated that TMA performs as well as full sections for immunocytochemical evaluation of malignancy and provides a high throughput approach. Concordance between IHC analysis of three TMA cores of FL and ten randomly cases for which full sections were also scored with the validation antibody panel was more than 90%. Prior to this study, very little work using the TMA technique had been performed on haematological malignancies and FL in particular. A single study in 2002 analysed a series of 193 B cell NHLs and 29 Hodgkins lymphoma and compared full section staining results to those obtained using TMA and found a high correlation (Hedvat CV et al. 2002). There were initial concerns that extracting small cores of FL might not include enough neoplastic follicles for analysis. To address this issue, the core diameter chosen was 1mm as compared to 0.6mm diameter which is more frequently chosen in other tissues such as breast cancer (Torhorst J et al. 2001). In addition three cores of tissue were extracted from every sample. The high concordance with full section evaluation has verified this

technique as an appropriate method for analysis of haematological malignancies. Subsequent studies have been performed on haematological malignancies and have demonstrated good correlation between full sections and tissue microarray cores (Garcia JF et al. 2003; Tzankov A et al. 2003; Zettl A et al. 2003).

Gene expression profiling of *de novo* DLBCL has allowed division of this disease into three different groups; germinal centre cell type (GC), activated B cell type (ABC) and type 3 with direct correlation to prognosis (Alidazeh AA et al. 2000). This genetic work has been translated to protein detection in tissue samples of *de novo* DLBCL using IHC (Hans CP et al. 2004). This work has demonstrated that FL transforms to DLBCL of GC phenotype in the majority of cases. This result is unsurprising as FL is a neoplasm of the germinal centre B lymphocytes. However, five year survival for the transformed patients from the time of transformation is only 38% despite the majority of these patients (89%) having a phenotype shown to be favourable in *de novo* DLBCL. It is unlikely that the cell of origin for these neoplasms is different as they both have the same phenotype. This may be a reflection of treatment effect. In patients with *de novo* DLBCL the disease is an acute initial presentation, they will have not received any prior treatment; however patients with transformed FL may have presented with FL many years before and have potentially received numerous therapies. Evidence indicates that administration of chemotherapeutic agents can produce alterations in cell biology in neoplastic cells which are not eradicated (Zaslav A-L, Stamberg J, Shende A 1988; Offit K et al. 1991; Liu Z-L et al. 2002). It is possible that genetic pathways increasing tumour aggressiveness have been activated whilst GC phenotype is retained. Patients with transformed FL will usually have been diagnosed for several years and consequently the tumour cells may have acquired numerous mutations which account for the increased tumour aggressiveness.

The confirmation of up-regulation of AURKB at protein level in a subset of patients is interesting as it is an attractive therapeutic target. Disruption of the AURKB kinase function in a t(14;18) DLBCL cell line results in apoptosis (Harrington EA et al. 2004). These results indicate that targeting the AURKB may be an effective therapeutic regime for some patients who transform from FL to DLBCL. The close correlation between gene transcript up-regulation and protein expression indicate that

immunocytochemistry is an efficient technique to identify those patients in whom AURKB directed therapies would be effective. This is valuable as gene expression analysis requires fresh tissue samples as well as being expensive and unavailable to clinicians who are not working in academic institutions with access to specialized laboratories. This study did not demonstrate a significant correlation with AURKB expression and survival. This may be a reflection of the small number of patients (n=31) with both pre-transformation and transformed samples available for analysis. In fact, within this disease entity and available from a single institution, this is a large series. In order to explore further any effect on survival larger sample numbers would be required possibly through collaborative studies with other institutions.

The up-regulation of gene transcripts and protein levels of nm23 on transformation is also interesting. Studies in other haematological malignancies have demonstrated a correlation between poor prognosis and increased nm23 protein levels (Niitsu N et al. 1999; Niitsu N et al. 2000). In DLBCL and peripheral T cell lymphoma the serum protein levels are an independent prognostic factor (Niitsu N et al. 2001). More interestingly, increased expression of nm23 in neoplastic tissue from patients with DLBCL correlated with reduced overall survival and progression free survival rates. Increased levels in the tissue corresponded with increased levels in serum (Niitsu N et al. 2004). This could prove to be a cost effective and less traumatic method of monitoring patients for evidence of transformation from FL to DLBCL as well as providing a clinical parameter for predicting survival. It may also provide an important target for therapeutic intervention, as the increase in serum levels of the protein in patients with positive tumours suggests that the lymphoma cells themselves may be producing nm23. The role of nm23 as a provider of high energy nucleosides (Parks RE Jr, Agarwal RP 1973) indicates that the tumours may be dependent on the provision of high energy compounds for survival thereby providing a potential selective therapeutic target. Unfortunately, nm23 levels are only elevated in a subset of patients on transformation. The high correlation between increase in gene transcript and detection of increased protein levels by immunocytochemistry identities immunocytochemistry as a cost effective and efficient method for selecting patients who may benefit from a compound targeting this molecule. Although there is no significant correlation between expression of nm23 and overall survival, this may be due to the small number of patients available for the study and may be

addressed by a larger collaborative study.

Immunocytochemistry for FOXP1 in *de novo* DLBCL demonstrated a slightly higher number of tumours with increased expression than the data produced by Hans et al. (2004) (73% versus 61%). Increased protein levels were observed in tumours with a non-GC phenotype. This is in contrast to the findings of a previous study where FOXP1 expression was present only in patients lacking a GC phenotype (Barran SL et al. 2004) but is in keeping with the data produced by Hans et al. (2004).

Finally, immunocytochemistry on TMA composed of samples from patients with transformed FL in association with the clinical history is a powerful tool for analyzing the protein changes occurring throughout the natural history of the disease. The ability to incorporate all biopsies from a single patient onto one slide removes all the procedural variables which might produce differences in immunocytochemical staining and increases confidence that any changes observed are true. It is also an important method of conserving valuable material, improving cost efficiency and increasing speed of analysis. This approach can aid the selection of patients who are most likely to benefit from directed therapeutic approaches.

CHAPTER FIVE:

Number of CD4-positive cells and location of FOXP3-positive cells in diagnostic follicular lymphoma tissue microarrays correlates with outcome

5.1 Introduction

The heterogeneous nature of FL makes the treatment of patients with this disease difficult. There are numerous treatment options available ranging from a simple 'watch and wait' strategy through various chemotherapeutic options to bone marrow transplantation. It is essential that patients are allocated to the most appropriate therapeutic option for them. Currently, this is based on varying clinical factors encompassed in the Follicular Lymphoma International Prognostic Index (FLIPI) (Solal-Celigny P et al 2004). Although the FLIPI is useful as an aid to stratify patients it has limited discriminative power and better prognostic biomarkers are needed. The observation that it was possible to predict survival in FL based on the molecular features of the TILs using gene expression profiling renewed interest in the role of the microenvironment in FL pathogenesis (Dave SS et al. 2004). There are disadvantages with gene expression profiling in that it is a technique which requires a level of expertise and equipment which may be beyond many general hospitals. In addition, it is expensive and requires fresh tissue for optimal results. Finally, the presence of these changes at a protein level in numerous samples would be a stronger indicator of importance than gene expression data alone.

5.1.1 Aims and Objectives**:**

In order to further explore and validate the gene expression data at a protein level a patient population with survival at the extremes of FL expected survival (<5 years and >15 years) was selected. Using paraffin-embedded diagnostic biopsies of FL, a TMA from 59 patients was constructed. The relationship between immune microenvironment phenotype and survival in FL was examined with particular regard to Tregs.

5.2 Materials and Methods

5.2.1 Patient samples

Ethical approval for this study was obtained from the Local Regional Ethics Board.

Thirty four initial diagnostic paraffin-embedded lymph node blocks samples from FL patients with survival of less than five years from diagnosis and 25 from patients whose survival was more than 15 years from diagnosis were identified from those treated at St. Bartholomew's Hospital between 1974 and 1999. Patients were treated according to the current protocol over the 25-year period (Gallagher CG et al.1986; Johnson P et al. 1995; Apostolidis J et al. 2000). Broadly, at presentation those with stage I disease were treated with involved-field irradiation, the remainder were managed expectantly, until chemotherapy was instituted when there was an indication for intervention. Following achievement of remission, management was again expectant.

5.2.2 Tissue microarray construction

Tissue microarrays (TMA) were constructed of 1mm cores of patient tissue taken from representative areas of lymphoma (>5 neoplastic follicles) using pre-marked H&E stained sections and from reactive tonsil and appendix controls, in triplicate using a manual arrayer (Beecher Scientific, Silver Spring, MD) as previously described (Hedvat CV et al. 2002; Kononen J et al. 1998). Approximately 120 cores were applied per slide.

5.2.3 Immunohistochemistry

The test panel of antibodies and dilutions used for immunohistological analysis are shown in **Table 7**. TMA blocks were cut to 4 microns and applied to TESPA-coated slides, then dewaxed and blocked in hydrogen peroxide/methanol solution. Antigen retrieval was performed by pressure-cooking in citrate, or in the case of FOXP3, EDTA (Norton AJ, Jordan S, Yeomans P 1994). Sections were then stained using the Vector Elite ABC kit (PK6100, Vector Laboratories, Peterborough, UK) or for FOXP3, CSA II tyramide amplification system (DAKO, Denmark), followed by diaminobenzidine chromogen (Biostat, Stockport, UK). Appropriate negative and positive controls were always used.

Table 7 Antibodies used for the investigation of the immune microenvironment

Test Antibody	**Clone**	**Source**	**Dilution**
CD4	NCL-CD4-368 clone4B12	Novocastra, Newcastle, UK	1/100
CD7	NCL-CD7-272 clone 272	Novocastra, Newcastle, UK	1/50
CD8	C8/144B	Dako	1/50
CD25	NCL-CD25-305 clone 4C9	Novocastra, Newcastle, UK	1/100
CD68	KP1	Dako	1/4000
Granzyme-B	NCL-GRAN-B clone 11F1	Novocastra, Newcastle, UK	1/40
TIA-1	Ab2712	Abcam, Cambridge, UK	1/50
FOXP3	Supernatant Clone 236A/E7	Gift from Alison Banham, John Radcliffe, Oxford (Roncador G et al. 2005)	Neat

5.2.4 Double staining for CD4 and FOXP3

After antigen retrieval by pressure cooking in citrate, staining for CD4 was performed using Vectastain Elite ABC kit with diaminobenzidine as chromogen.

This was followed by staining for FOXP3 using Vectastain Elite ABC-AP (alkaline phosphatase) with Fast Red as chromogen.

5.2.5 Immunohistochemical analysis

Presence of adequate FL tumour cells in the tissue cores was confirmed by scoring with a diagnostic lymphoma antibody panel comprising CD3, CD5, CD10, CD20, CD21, CD23, BCL-2, BCL-6, Ki-67, TP53, MUM-1 as previously described in Chapter 4 (data not shown).

The entire 1mm diameter core was analysed at low power (x5 magnification) and high power (x40 magnification) in each case. Where one of three cores could not be scored due to insufficiency of tissue, consensus of 2 cores was accepted although this was necessary in only 10% of the total 1,416 cores analysed. If two cores of three were insufficient, no scoring was attempted and the result was noted as 'absent' for that sample with the antibody in question. As a result, the maximum number of patient cases (34 for the short-survival group and 25 for the long-survival group) were not always assessable for each of the test panel antibodies.

Staining using a test panel of immune-cell-associated antigens CD4, CD7, CD8, CD25, CD68, TIA-1, Granzyme B and FOXP3 was evaluated for number of positive cells. Scoring was divided into the following categories, <5 cells/high power (x40 magnification) field (hpf), 5-10 cells/hpf, 10-15 cells/hpf, 15-30 cells/hpf and >30 cells/hpf. Topographical distribution of positive cells was also evaluated. Location relative to the neoplastic follicle (perifollicular, intrafollicular and not bearing either of those patterns but being evenly distributed) was recorded.

5.2.6 Statistical analysis

Fisher's Exact Test was performed to determine whether the incidence of antigen expression was significantly different between samples from patients at the extremes of survival

5.3 Results

5.3.1 Patient Characteristics

Diagnostic FL biopsies from patients at the extremes of survival were studied in this

work (Lee AM et al. 2006). Histology was reviewed in all cases. The characteristics of the 59 patients are shown in **Table 8**. Patients who lived less than 5-years from diagnosis, the 'short survivor group', had a median survival of 2 years and all died of disease. Patients who lived more than 15 years from diagnosis, the 'long survivor group' had a median survival of 21 years. Although the short survivor group, were significantly older than the long survivor group with a median age of 61 and 46 years respectively, as shown in **Table 8**, there was no significant difference in the incidence of transformation, of bone marrow involvement, stage or grade between these two patient groups.

Table 8 Demographics of patients from the extremes of survival

Characteristics		**'Short Survivor Group'**	**'Long Survivor Group'**
N=59		34 (25 male, 9 female)	25 (16 male, 9 female)
Median survival (years)		2 (0.08-4.8)	21 (15.3-30.4)
Median age at Diagnosis		61 years (range 28-80 years)	46 years(range 24-63 years)
Stage at diagnosis	I	4 (12%)	3 (12%)
	II	3 (9%)	6 (24%)
	III	4 (12%)	4 (16%)
	IV	23 (68%)	12 (48%)
Histological pattern		Follicular: 33 (97%) Follicular and Diffuse: 1 (3%)	Follicular: 24 (96%) Follicular and Diffuse: 1 (4%)
Grade at diagnosis	1	18 (53%)	16 (64%)
	1&2	3 (9%)	4 (16%)
	2	8 (24%)	2 (8%)
	2&3	1 (3%)	3 (12%)
	3	4 (12%)	0
	3b	0	0
Proceeded to Transform		9/34 (26%)	3/25 (12%)
Time to Transformation		2 at 1-yr, 2 at 2-yrs and 5 at 3-yrs	2, 7 and 16-yrs
Cause of death		Disease: 34 (100%)	Still alive: 15 (60%) Disease: 3 (12%) Other disease or unrelated infection: 7 (28%)

Bone marrow involvement		17/34 (50%)	12/25 (48%)
Treatments			
	Exp.Management	1 (3%)	1 (4%)
	1	7 (21%)	6 (24%)
	2	6 (18%)	6 (24%)
	3	4 (12%)	3 (12%)
	4	5 (15%)	3 (12%)
	5	2 (6%)	2 (8%)
	6	6 (18%)	0
	7	3 (9%)	1 (4%)
	8	0	3(12%)

5.3.2 Analysis of the TMAs

For each antibody, 2 (maximum of 10% cases over all the antibodies studied) or 3 replicate cores were assessable from 31 to 34 cases from the short-survival group and 22 to 25 cases from the long-survival group. Scoring was also divided into the following categories, <5 cells/hpf, 5-10 cells/hpf, 10-15 cells/hpf, 15-30 cells/hpf and >30 cells/hpf. Analysis of these categories did not reveal any additional correlation with outcome and for further analysis the categories <5 cells/hpf and >5 cells/hpf were used (**Table 10**).

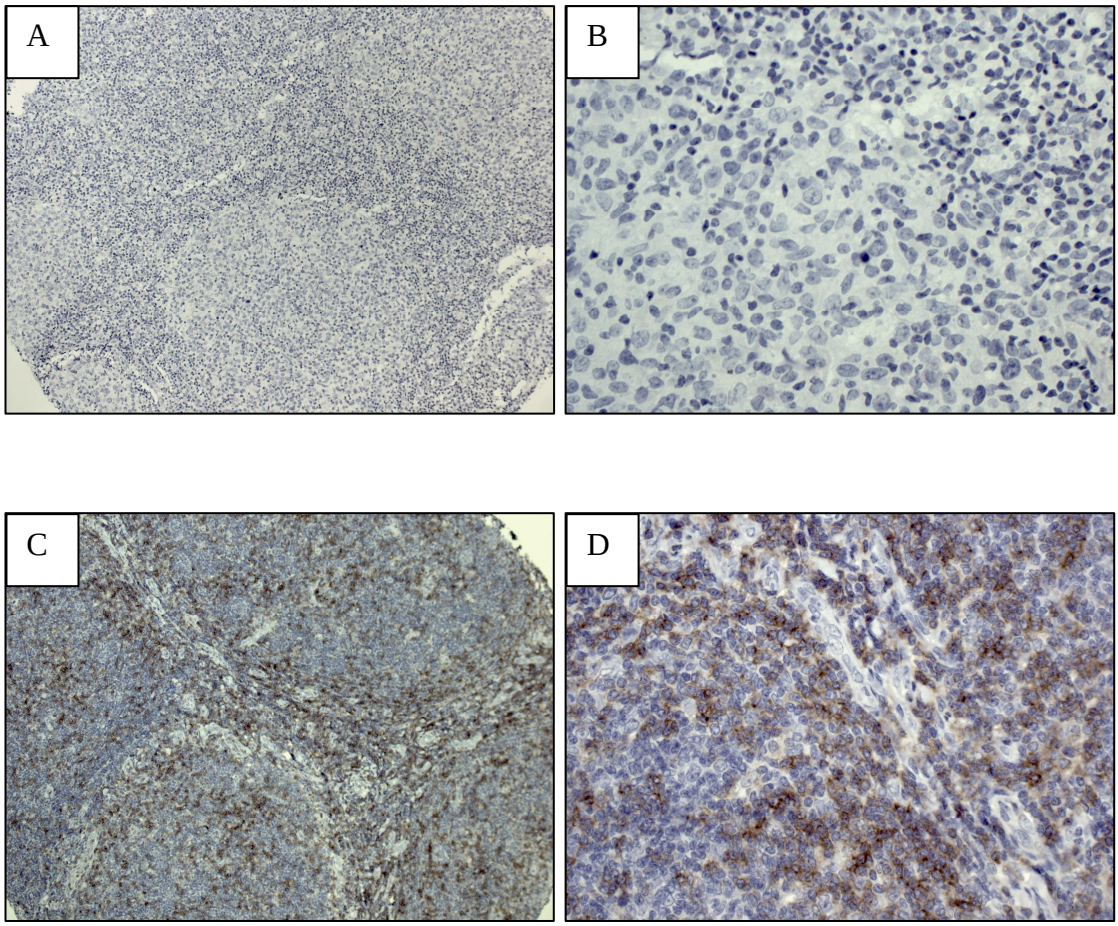
5.3.3 CD4; Incidence is significantly different between short and long survivor groups

CD4 expression was assessable in 56 out of 59 (95%) total cases; the 3 unassessable cases were all from the long survivor group. CD4-expressing cells were observed at levels >5/hpf in 36/56 (66%) cases; 18/34 (53%) cases from the short survivor group and 18/22 (82%) biopsies from the long survivor group (p=<0.05; **Table 9**). Representative CD4 stained TMA cores are shown in **Figure 35.**

Table 9 Expression of immune cell antigens in diagnostic FL lymph nodes

Antigen	**Number of assessable cases (N=)**		**<5 cells positive/high power field**		**>5 cells positive/high power field**	
	Short Survivor Group	**Long Survivor Group**	**Short Survivor Group**	**Long Survivor Group**	**Short Survivor Group**	**Long Survivor Group**
CD4	34	22	16/34 47%	4/22 18%	18/34 53%	18/22 82%
			P=<0.05			
FOXP3	31	24	11/31 35%	3/24 13%	20/31 65%	21/24 87%
			P=0.07			
CD8	33	22	3/33 9%	0/22 0%	30/33 91%	22/22 100%
			P=0.27			
CD7	34	23	6/34 18%	1/23 4%	28/34 82%	22/23 96%
			P=0.22			
CD25	33	23	11/33 33%	4/23 17%	22/33 67%	19/23 83%
			P=0.23			
CD68	33	22	3/33 9%	2/22 9%	30/33 91%	20/22 91%
			P=1.00			
TIA-1	32	24	3/32 9%	1/24 4%	29/32 91%	23/24 96%
			P=0.63			
Granzyme B	31	25	24/31 77%	20/25 80%	7/31 23%	5/25 20%
			P=1.00			

Figure 35 CD4 expression on TMA cores from diagnostic FL biopsies taken at low power (OMx 10 magnification) (A, C) and high power (OMx 40 magnification) (B, D). Cases with absence (<5 cells / high power field) (A, B) and presence (C, D) of CD4 expression (>5 cells/high power field) are illustrated.



The incidence of a very high level of expression of CD4 (>30 cells/hpf) was the same between the two groups (**Table 10**).

Table 10 Incidence of CD4 + cells in the short and long survivor groups of FL demonstrating no advantage in smaller divisions of cell number

Cell no. /high power field	**Short Survivor Group (number of cases)**	**Long Survivor Group (number of cases)**	**Difference between the two survivor groups**
<5	16	4	p=0.045
5-10	3	6	p=0.133
10-15	3	2	p=1.000
15-30	6	5	p=0.736
>30	6	5	p=0.736
Total cases	34	22	

Amongst all the patients studied (56 assessable for CD4) those with <5 CD4 cells/hpf in their diagnostic sample had a median age of 62-years (range 34-77 years old) while the median age of those bearing >5 CD4 cells/hpf was 54-years (range 24-80 years old) (p=0.08).

We examined the location of positively staining cells in relation to the neoplastic follicle (intrafollicular, perifollicular and evenly distributed with no intra- or perifollicular pattern). Absence of CD4 correlated with poor outcome (p=<0.05, Table 8), and in cases where CD4-positive cells were present, there was a trend towards a significant difference in their location between the two survivor groups (Fishers exact 3 x 2, p=0.09; **Table 11**).

Table 11 Location of CD4+, CD25+, or FOXP3-Positive cells in short and long survival groups with FL, and correlation with survival

	CD4		**CD25**		**FOXP3**	
Location of positive cells	**No. of Short Survivor Cases/34**	**No. of Long Survivor Cases/22**	**No. of Short Survivor Cases/33**	**No. of Long Survivor Cases/23**	**No. of Short Survivor Cases/31**	**No. of Long Survivor Cases/24**
Absence	16 (47%)	4 (18%)	11 (33%)	4 (17%)	11 (35%)	3 (13%)
Perifollicular	10 (29%)	11 (50%)	3 (9%)	7 (30%)	2 (6%)	8 (33%)
Throughout	8 (24%)	7 (32%)	19 (58%)	12 (52%)	18 (58%)	13 (54%)
Difference in distribution between the survivor groups Fisher's Exact	p=0.09		p=0.1		p=0.02	

Key

Absence: <5/hpf

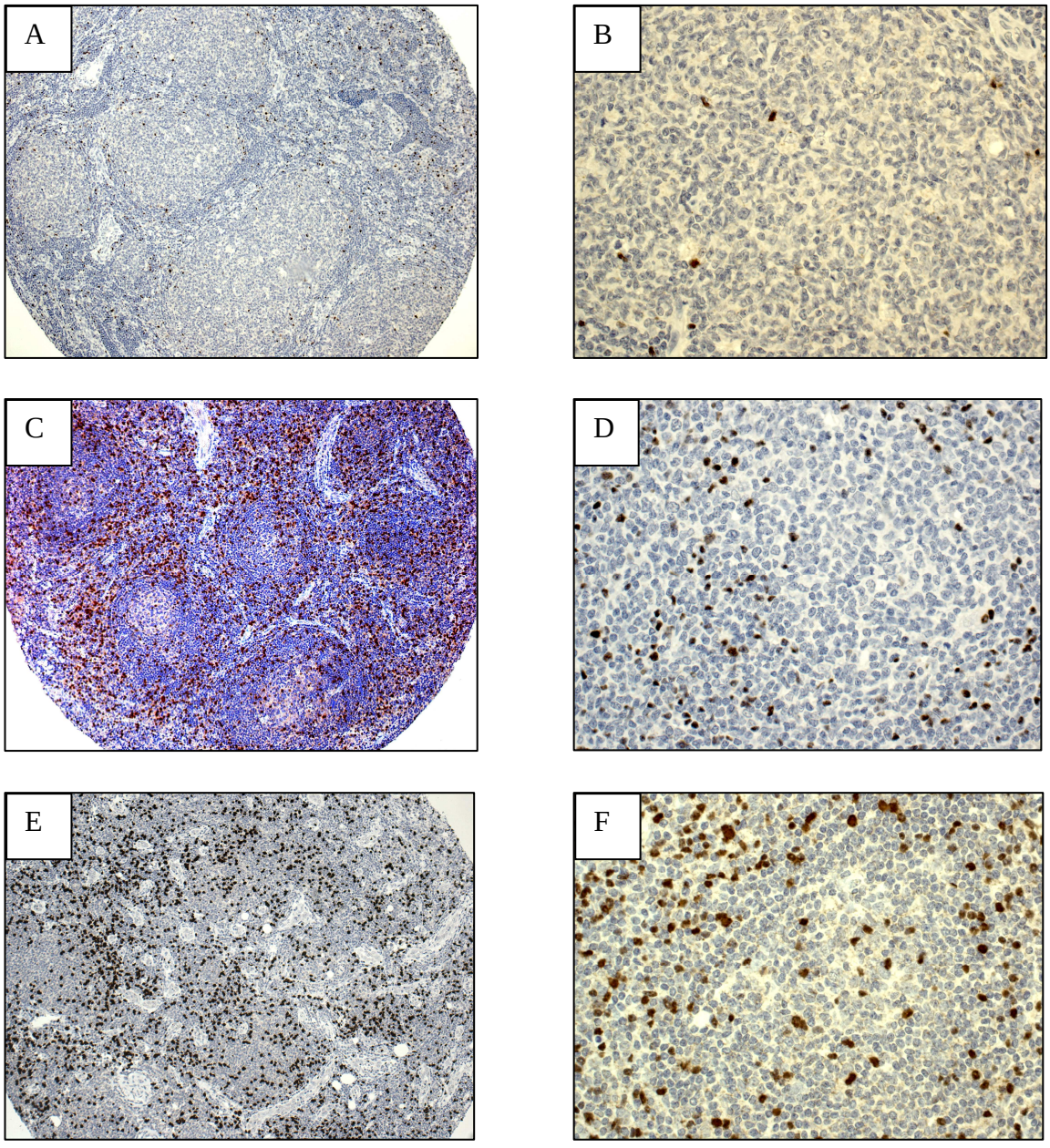
Perifollicular: intrafollicular <5/hpf; perifollicular 5-30/hpf

Throughout: positive cells (>5/hpf) evenly distributed across the cores and no clear perifollicular pattern observed

5.3.4 T regulatory cells and outcome

There was a trend towards patients with long survival being more likely to contain FOXP3 positive cells in their biopsy (p=0.07) but none of the other immune cell associated antigens studied showed any significant association between frequency and survival (**Table 9**), In addition, the pattern of FOXP3 expression was significantly different in the short and long survivor groups (Fishers Exact 3 x 2 p=0.02; **Table 11**). Examples of the different staining patterns observed for FOXP3 expression are shown in Figure 36. Patients from the long survivor group had FOXP3-positive cells at a higher incidence in a perifollicular location than the patients of the short survivor group (collapsed 2 x 2 Fisher's Exact p=0.01; **Table 12**).

Figure 36 FOXP3 expression on TMA cores from diagnostic FL biopsies taken at low power (OM x10) (A, C, E) and high power (OM x40) (B, D, F). Cases with absence (<5 cells / high power field) (A, B) and presence of FOXP3 expression (>5 cells/high power field) in a perifollicular pattern (C, D) or evenly distributed throughout the tissue (E, F) are illustrated.



The location of CD25 expression showed a trend towards occurrence in a perifollicular location amongst the samples from patients within the long survivor group (collapsed 2x2 Fisher's Exact p=0.07; **Table 12**)

Table 12 Locations of CD4, CD25 or FOXP3-positive cells in short and long survivor groups with FL, and correlation with survival

	CD4		CD25		FOXP3	
	No. of Short Survivor Cases/34	**No. of Long Survivor Cases/22**	**No. of Short Survivor Cases/33**	**No. of Long Survivor Cases/23**	**No. of Short Survivor Cases/31**	**No. of Long Survivor Cases/24**
Absence+throughout	24	11	30	16	29	16
Perifollicular	10	11	3	7	2	8
Difference in distribution between the survivor groups. Fisher's Exact	p=0.16		p=0.07		p=0.02	
Absence+perifollicular	26	25	14	11	13	11
Throughout	8	7	19	12	18	13
Difference in distribution between the survivor groups. Fisher's Exact	p=1.00		p=0.79		p=0.59	

Key

Absence: <5/hpf

Perifollicular: intrafollicular <5/hpf; perifollicular 5-30/hpf

Throughout: positive cells (>5/hpf) evenly distributed across the cores and no clear perifollicular pattern observed

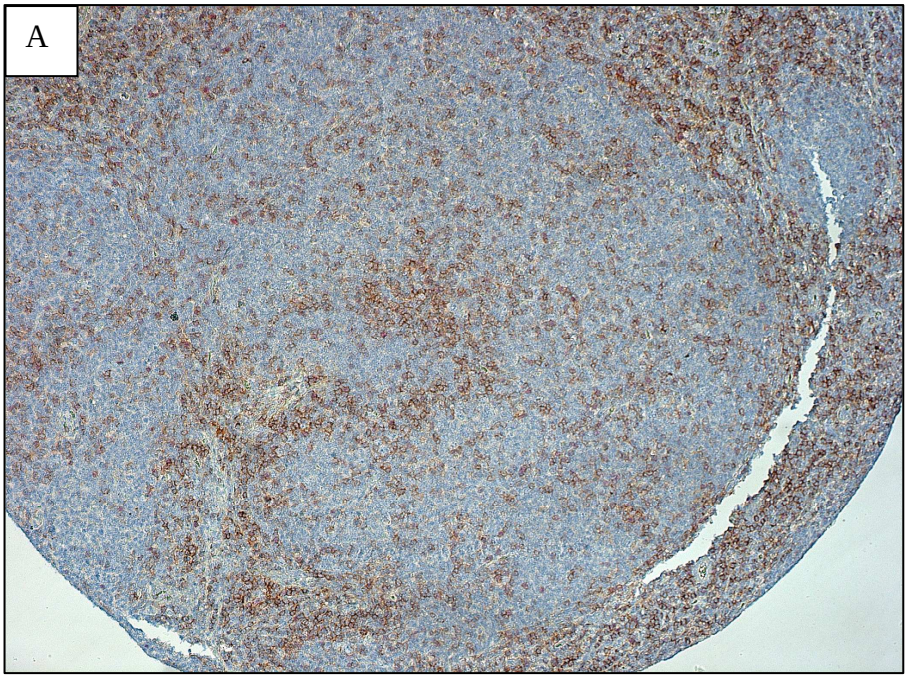
The finding that CD4 incidence and FOXP3 distribution at diagnosis were important to outcome implied the involvement of the Treg subset that variably bear these antigens. The incidence of cells expressing CD4, CD25 or FOXP3 on the same cases from the short survivor group (n=30) and the long survivor group (n=20) is shown in **Table 13**.

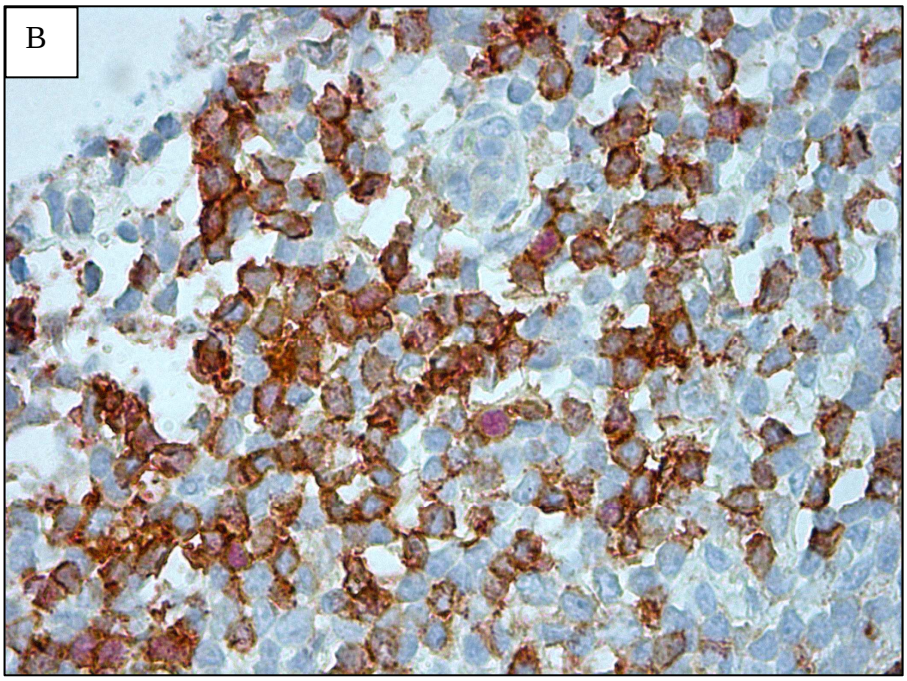
Table 13 Incidence of CD4, CD25 and of FOXP3 in same diagnostic biopsies of short and long survivors of FL

	Short Survivor Group			**Long Survivor Group**		
Cell no. /high power field	**CD4**	**CD25**	**FOXP3**	**CD4**	**CD25**	**FOXP3**
<5	14	9	10	5	3	3
5-10	2	4	8	3	10	8
10-15	3	7	7	2	6	7
15-30	6	7	4	5	1	1
>30	5	3	1	5	0	1
Total cases	N=30			N=20		

Double staining with CD4 and FOXP3 revealed cells co-expressing these antigens in a case from the long survivor group (**Figure 37 (A) and (B)**)

Figure 37 Double staining for CD4 (membranous brown) and FOXP3 (nuclear red) Long survivor-group – A) low power (OM x10), B) high power (OM x63)





5.4 Discussion

This study of 59 diagnostic samples taken from FL patients whose outcome was either good or poor draws attention to the importance of the immune system in the setting of haematological malignancy and provokes speculation as to the roles and mechanisms involved. We identified that CD4-positive cells and a perifollicular pattern of FOXP3-positive cells are more commonly found in the diagnostic FL lymph node from patients whose subsequent outcome was favourable. These findings are in keeping with the hypothesis that the presence of CD4-positive cells are of benefit to the host. This has been previously observed in a study where significantly more CD4-positive T lymphocytes were present in the biopsies of untreated FL patients who underwent spontaneous remission (Strickler S et al. 1988).

The finding that the presence of CD4 T lymphocytes is more commonly observed in the biopsies from patients with an improved prognosis is in keeping with the gene expression data (Dave SS et al. 2004). Although CD7 appears as a member of the immune response gene expression signature correlating with better outcome (IR-1) in FL (Dave SS et al. 2004) expression of CD7 protein alone did not correlate with survival in the data presented here. The data generated by Farinha and colleagues using immunohistochemistry for CD68, a macrophage marker, observed a short survival correlated with >15 positive staining cells/hpf in diagnostic biopsies (Farinha P et al. 2005). Immunocytochemical expression of CD68 was used as a surrogate for the macrophage IR-2 signature. In the patient groups examined in this work no correlation was found between the numbers or the location of CD68 positive cells and survival. This may be a reflection of the very different patient populations studied, the patient group studied by Farinha et al. was composed of younger patients (median age 44 years range 19 – 61 years) with aggressive disease treated with multi-agent chemotherapy (Farinha P et al. 2005). Alternatively it may result because CD68 is not one of the 24 genes in the IR-2 signature (Dave SS et al. 2004) and other genes from that signature should be assessed.

It is known that age-related changes in the immune system occur; function is impaired as exemplified by reduced vaccination efficacy (Cook JM et al. 1987; Phair J et al. 1978) and by higher incidence of certain infections, cancers and autoimmune disease (Yoshikawa TT 2000). While absolute numbers of lymphocytes are

maintained throughout life (Hulstaert F et al. 1994; Pido-Lopez J et al. 2002) the T-cell subset composition and distribution may alter (Wallace DL et al. 2004). The frequency of CD4-positive cells in the FL lymph nodes showed a trend towards correlation with age in the present study. A larger sample size may therefore demonstrate that the adverse relation between age and outcome in FL is associated with age-related changes in the immune cell composition.

In non-neoplastic lymphoid tissue, distribution of FOXP3-positive cells varies. Within tonsils FOXP3 cells are in a predominantly perifollicular distribution whereas within reactive lymph nodes an evenly distributed pattern is seen (Roncador G et al. 2005). The data from this study shows FOXP3-positive cells occur in the perifollicular region more commonly in samples from patients with longer survival. This would seem to differ from data in epithelial malignancies where recruitment of FOXP-3 positive Tregs within the tumour appears to promote tumour tolerance (Curiel TJ et al. 2004). FL is a malignancy of the haematopoietic system from which the Tregs are an integral part and so their impact on tumours of this system may be very different. It has been shown that activated CD4+CD25+ T lymphocytes can kill B lymphocytes in a cell-contact dependent manner, induction of death is caused by up-regulation of perforin and granzyme in the CD4+CD25+ T cells (Zhao DM et al. 2006). CD4+CD25+ T cells have also been shown to lyse antigen presenting B cells by Fas-Fas ligand interaction (Janssens W et al. 2003). CD4+CD25+ T cells can down-regulate the expression of the co-stimulatory molecules CD80 and CD86 on dendritic cells (Cederbom L, Hall H, Ivars F 2000). In addition, regulatory T cells can suppress immunoglobulin production and class switch recombination by direct action on B lymphocytes (Lim HW et al. 2005). All of these actions of Tregs can act on neoplastic B lymphocytes which comprise FL and prevent its effective survival and expansion. The findings in this study are in keeping with others in haematological malignancies. Carreras et al. recently reported that a high number of FOXP3-positive cells correlated with improved survival in FL although the location of these cells was not important (Carreras J et al. 2006). Minor differences in their study and this study may be explained by variance in samples and methodology. Carreras et al. used computer-analysis of full sections, studied more patients with a mixed and diffuse histology and defined location differently (Carreras J et al. 2006).

In classical Hodgkins disease (cHD) an inverse relationship between TIA-1 and FOXP3 expression and survival has been demonstrated; higher levels of FOXP3 twinned with low TIA-1 being beneficial (Alvaro T et al. 2005). Analysis of TIA-1 and FOXP3 in this study of FL did not demonstrate the same findings. FOXP3-positive cells are the rate limiting subset of the immune response. Their presence surrounding the highly active germinal centre in an infected tonsil would support this function and leads to speculation that patients who do well after diagnosis of FL may do so in part because of an active immune response at the time of developing the tumour, with the lymphoma cells either as target or as bystander.

The Treg-associated markers CD4, CD25 and FOXP3 did not consistently correlate, most likely because these antigens are variably expressed. It is known there is a subset of CD8 Tregs (Xystrakis E et al. 2004). Additionally, in humans FOXP3 positive cells can be $CD25^{high}$ or $CD25^{low}$ (Fontenot JD et al. 2005a).

The prognostic significance of the immune microenvironment in FL at diagnosis may reflect factors involved in determining the inherent ‘aggression’ of the tumour and its ability to recruit infiltrating cells on the one hand or co-exist with naturally occurring cells of the lymph node on the other. This study has identified that CD4-expressing cells and perifollicular distribution of FOXP3-expressing cells occurs more commonly in the diagnostic tumour microenvironment of FL patients with better outcome compared to those with poor outcome. Elucidating the phenotype and functional capabilities of these cells is likely to provide a better understanding of the impact of the immune microenvironment on tumour progression in FL and may identify novel targets for the potentiation of tumour immunotherapy. The findings in this current study can be easily translated to the routine diagnostic histopathology setting and may be a method to aid identification of patients with potentially aggressive disease at the time of diagnosis.

CHAPTER SIX:

Immunophenotyping of Regulatory T lymphocytes in the Peripheral Blood

6.1 Introduction

CD4+CD25+ regulatory T lymphocytes isolated from human peripheral blood suppress the proliferation and cytokine production of naïve and memory T cells in vitro.

Two different isoforms of *FOXP3* have been identified in CD4+CD25+ Tregs with one version lacking the second coding exon. Human CD4+CD25+ Tregs co-express the two isoforms of *FOXP3* and it is only when both isoforms are expressed is there consistent and strong suppression of IL-2 and IFN-γ production (Allan SE et al. 2005).

Previous work has demonstrated an increase in CD4+CD25+ T cells with Treg characteristics in the peripheral blood of patients with epithelial malignancies as compared to healthy controls. These CD4+CD25+ cells were able to suppress the proliferation of CD4+CD25- T cells and could inhibit NK-cell-mediated cytotoxicity (Wolf AM et al. 2003).

The aim of this work was to determine the proportion of CD4 positive Tregs within the PBMC of healthy controls and patients with FL. In addition, the number of CD8 positive Tregs in healthy controls and patients was assessed and a more detailed phenotype of Tregs performed by flow cytometry. There are reports of Tregs which are CD25 negative (Fontenot JD et al. 2005). FOXP3 expression has been demonstrated to be transiently up-regulated in a small proportion of CD4+ and CD8+ cells upon in-vitro stimulation. These cells were unable to suppress T helper 1 cytokine expression (Gavin MA et al. 2006). In a non-suppressive cell population FOXP3 expression was demonstrated to be transient (Wang J et al. 2007). Recent studies have identified the α chain of the IL-7 receptor, CD127, to be down-regulated on cells expressing FoxP3 and exhibiting suppressive activity (Liu W et al. 2006; Seddiki N et al. 2006). Consequently CD127 was included in flow cytometry to ensure that true regulatory cells were assessed.

6.2 Materials and Methods

Ethical approval was sought and approved by the East London and The City HA Local Research Ethics Committee 3; REC reference number 05/Q0605/140. Frozen peripheral blood mononuclear cells (PBMCs) from pre-treated patients with confirmed FL were obtained from the Tissue Bank maintained by the Medical Oncology Unit of St Bartholomew's Hospital. Fresh samples were also obtained from patients in clinic. Fresh peripheral blood was also obtained from healthy controls. All patients and controls consented to the storage and use of their samples for ethically approved research.

6.2.1 Quantification of the percent of regulatory T cells in fresh PBMC

Peripheral blood mononuclear cells from patients with FL and healthy controls were separated from whole blood by ficoll-hypaque density gradient. The monolayer was removed and the cells washed and a cell count performed after resuspension in 5mLs of washing buffer. Aliquots of 1 million cells were removed for flow cytometry analysis at this point and stored on ice.

6.2.2 Thawing of cryopreserved cells

The vial was removed from liquid nitrogen and thawed immediately in a water bath at 37˚C and transfer to an empty sterile 15mL Falcon tube. RPMI medium was added dropwise to a volume of 10mL. The tube was centrifuged at 393g for 10 minutes at room temperature. The supernatant was removed and the pellet resuspended in 5mLs of RPMI and the dead cells separated using ficoll-hypaque density gradient. The monolayer was removed and the cells washed and a cell count performed after resuspension in 5mLs of washing buffer. Aliquots of 1 million cells were removed for flow cytometry analysis at this point and stored on ice. We noted no differences between fresh and frozen samples.

6.2.3 Flow cytometry

One mL of degassed buffer was added to each aliquot and the samples centrifuged at 393g for 10 minutes at 4-8ºC. Ten µL of each required antibody was then added to the appropriate tubes and the samples incubated for 30 minutes at 4ºC. The cells were than washed in degassed buffer and centrifuged at 393g for 10 minutes at 4ºC. If required, intracellular staining for FoxP3 was then performed. The supernatant was

removed and the cells resuspended in 500μL of degassed buffer. The antibodies and conjugates used are indicated in **Table 14**.

Table 14 Antibodies used for Flow Cytometry

Antigen	Conjugate	Amount (μL)	Dilution	Isotype	Manufacturer
CD3	APC	10	1:11	IgG2a	Miltenyi Inc
CD4	FITC	10	1:11	IgG2a	Miltenyi Inc
CD8	FITC	10	1:11	IgG2a	Miltenyi Inc
CD19	APC	10	1:11	IgG1	Miltenyi Inc
CD25	PE	10	1:11	IgG2b	Miltenyi Inc
CD127	PE	20	1:10	IgG1	eBioscience
FOXP3	APC	10	1:11	Mouse IgG1	Miltenyi Inc
Isotype control	FITC	10	1:11	Mouse IgG2a	Miltenyi Inc
Isotype control	APC	10	1:11	Mouse IgG1	Miltenyi Inc
Isotype control	PE	10	1:11	Mouse IgG2a	Miltenyi Inc
Isotype control	PE	20	1:11	Mouse IgG1	Miltenyi Inc

Flow cytometry was performed on a LSR cytometer (BD Biosciences, Cowley, Oxford). FlowJo software (Treestar Inc) was used for analysis. The initial voltage settings were obtained using an unlabelled cell sample. The unlabelled cell sample was also used to draw a gate over the lymphocyte region as determined by forward (FSC) and side scatter (SSC). Compensation levels between FL1 (FITC) and FL2 (PE) were set using cell samples stained with a single fluorochrome.

6.2.3.1 Identification of regulatory T cells

The lymphocyte gate determined using the unlabelled cell sample was applied to the

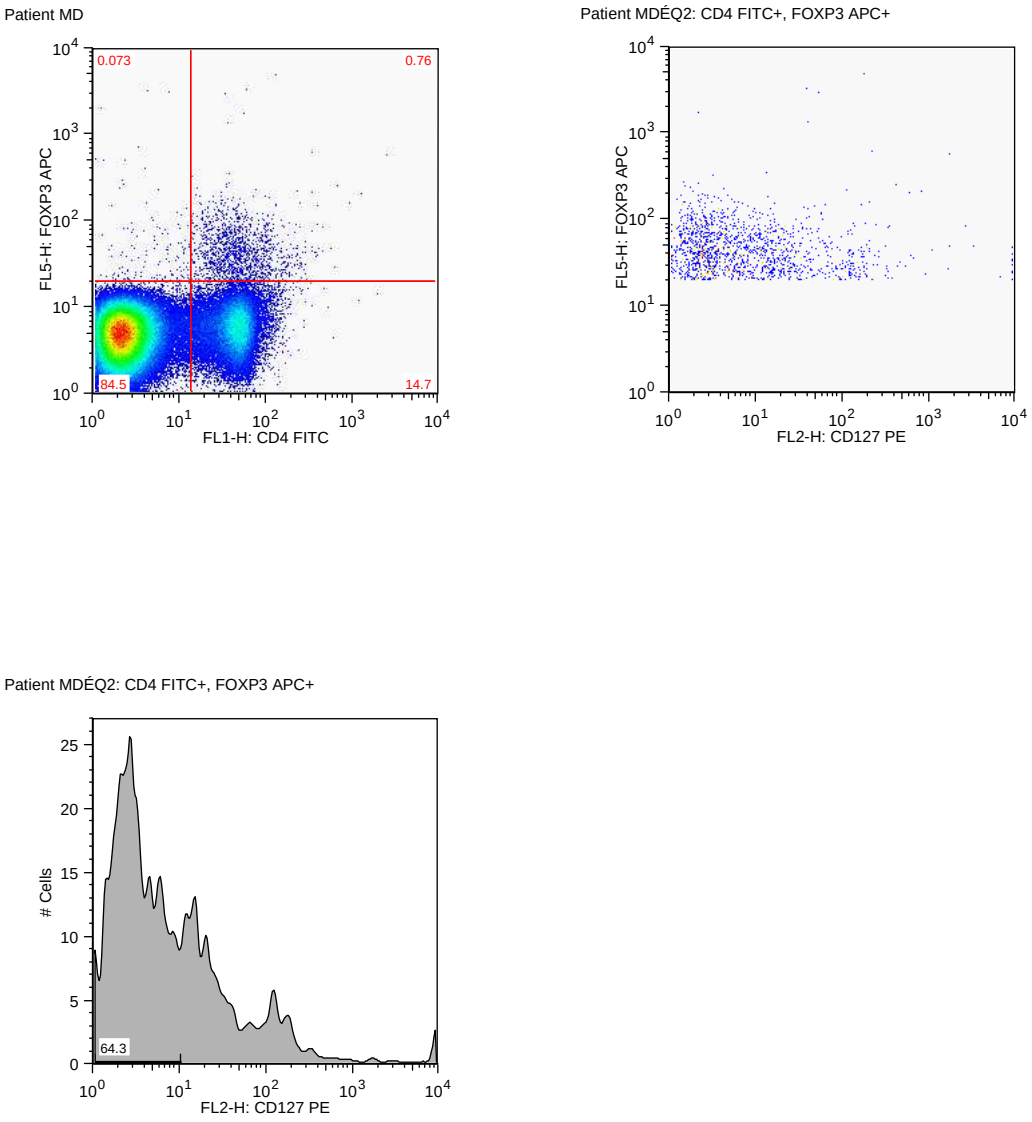
stained samples. Within this gate the CD4 or CD8 positive cells were identified using the isotype control as a gating measure. The CD4 or CD8 positive cells were selected and those positive for FOXP3 were identified using the isotype control as a gating measure. Finally the CD4/FOXP3 positive or CD8/FOXP3 positive cells were selected and the amount of CD127 positivity assessed. Those cells which were negative for CD127 were designated as regulatory T cells.

6.3 Results

6.3.1 The use of FOXP3 and CD127 identifies a smaller population of Tregs than use of CD4 and FOXP3

As described in the Materials and Methods (6.2.2.1) Tregs were identified using both FOXP3 positivity and negativity for CD127. The use of both of these markers identified a population of cells which was smaller than the population identified using FOXP3 positivity. This demonstrated in **Figure 38** below; the first graph shows a CD4 and FOXP3 positive population of 0.76%. The expression of CD127 in this population demonstrates that not all of these cells are CD127 negative. Using the histogram setting 64.3% of this cell population were identified as CD127 negative therefore the percent of Tregs which are CD4+FOXP3+ and CD127 negative is 64.3% of the originally identified percentage

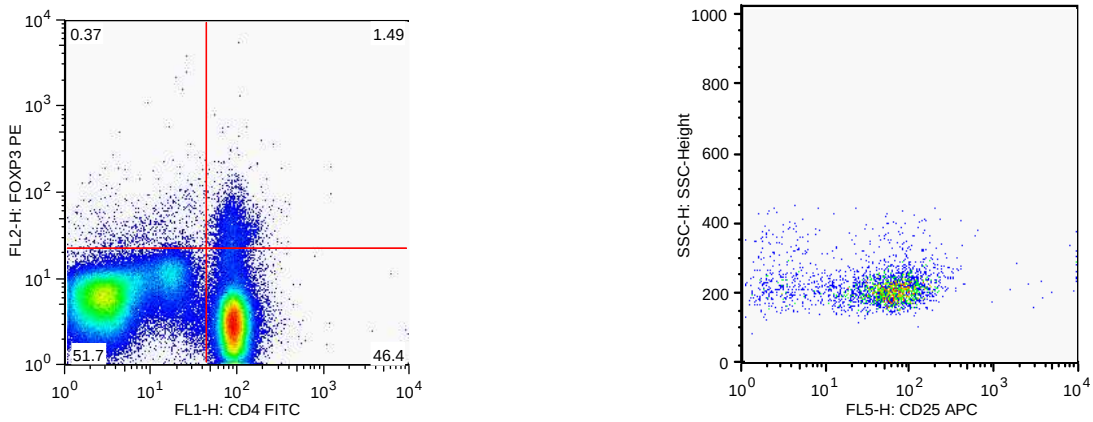
Figure 38 Flow cytometry graphs demonstrating that FOXP3 and CD127 together identify a smaller population of Tregs than FOXP3 staining alone



6.3.2 CD4 positive FOXP3 positive cells are not all CD25 high

The expression of CD25 in the CD4+FOXP3+ cell population was explored. The CD25 expression of this cell population was demonstrated to be predominantly high although a reasonable population of these cells were low and medium (**Figure 39**). This finding agrees with previous data (Fontenot JD et al. 2005).

Figure 39 Flow cytometry plots demonstrating the variable expression of CD25 in the CD4 positive FOXP3 positive cell population



6.3.3 Phenotypic analysis of peripheral blood mononuclear cells in healthy controls compared to patients with FL

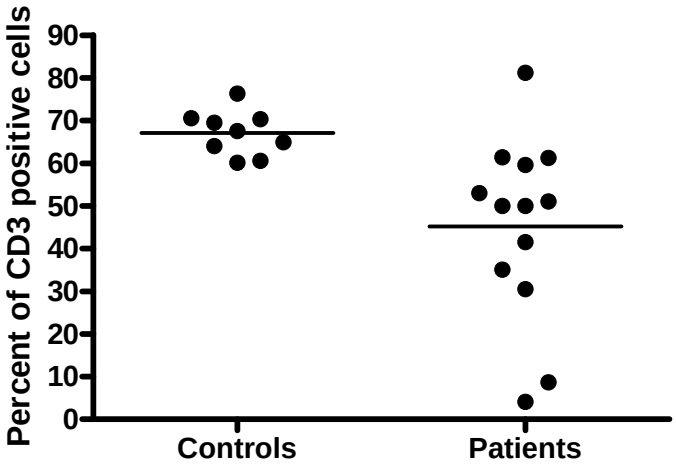
Phenotypic analysis was performed on the lymphocyte population of peripheral blood mononuclear cells of 13 age matched healthy controls and 18 pre-treated patients with follicular lymphoma. The mean percentage of the lymphocyte gate CD3 positive cells in healthy controls was significantly higher (67.08%) than the percentage of CD3 positive cells in patients with FL (45.18%) (p=0.0074) (**Figure 40**). The treatment regimes of the patients are listed in **Table 15**

Table 15 Clinical treatment and status of patients at time blood sample taken

Patient	**Diagnosis**	**Age at diagnosis (years)**	**Treatment**	**Status at time sample taken**
1	FL	35	CHOP	Expectant management at first relapse
2	FL	59	Radiotherapy	CR post treatment
3	FL	27	Transplant	Remission post transplant
4	FL	34	CVP	Expectant management at fourth relapse
5	FL	56	Expectant management	Expectant management
6	FL	50	Velcade and rituximab	Fourth relapse initial GPR
7	FL	37	Transplant	CR post transplant
8	FL	27	Transplant	CR post transplant
9	FL	60	Transplant	CR post transplant
10	FL	67	Expectant management	Expectant management
11	FL	32	Chlorambucil	GPR
12	FL	46	Transplant	CR post transplant
13	FL	81	Radiotherapy	Expectant management at first relapse

14	FL	34	FMD	CR
15	FL	40	Transplant	Expectant management at sixth relapse

Figure 40 Percent of CD3 positive cells peripheral blood mononuclear cells in healthy controls compared to patient samples from patients with FL



In addition, the variability of percentage of CD3 positive cells was significantly higher in the patient population compared to the healthy controls (p=0.0005).

6.3.4 Phenotypic analysis of regulatory T lymphocytes in PBMC from healthy controls and patients with FL

There was no significant difference between the percent of CD4 Tregs defined as CD4+FOXP3+CD127-, expressed as a percentage of the total CD4 positive cell population, in the patient samples (3.57%) compared to the healthy control samples (1.84%)(p=0.24)(**Figure 41**). The variation between patient samples and healthy controls was statistically significant (p=0.0001) (Figure 42).

Figure 41 Percent of CD4 regulatory T cells expressed as a percentage of total CD4 positive lymphocytes in the peripheral blood of patient samples from patients with FL compared to healthy controls

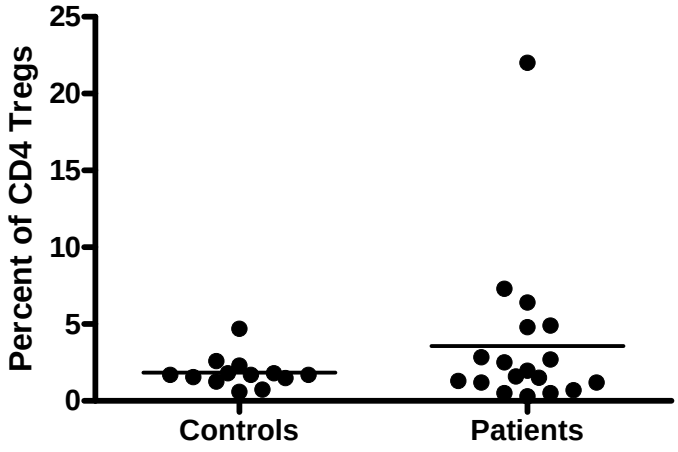
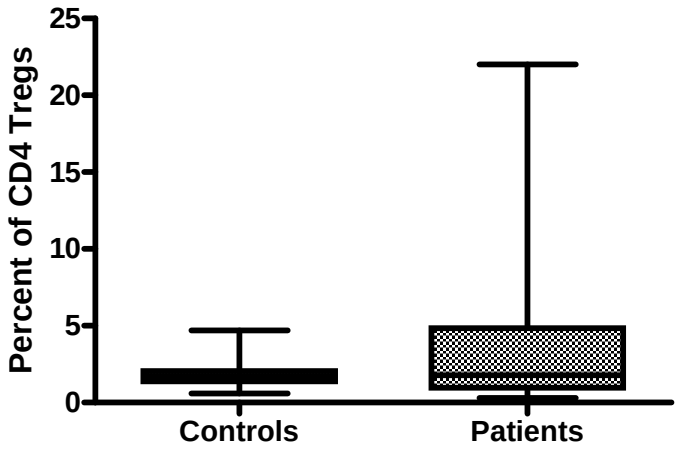


Figure 42 Graph to demonstrate the variance in percent of CD4 regulatory T cells expressed as a percentage of total CD4 positive lymphocytes in the peripheral blood of patient samples from patients with FL compared to healthy controls



As expected, there were fewer numbers of CD8 positive Tregs. There was no significant difference between the percent of CD8 positive regulatory T cells expressed as a percentage of the total CD8 positive cell population, in the peripheral blood mononuclear cell patient samples from patients with FL (0.34%) as compared to the samples from healthy controls (0.33%) (p=0.93)(**Figure 43**). There was no significant variation between samples in patients or healthy controls (**Figure 44**).

Figure 43 Percent of CD8 regulatory T cells expressed as a percentage of total CD8 positive lymphocytes in the peripheral blood of patient samples from patients with FL compared to healthy controls

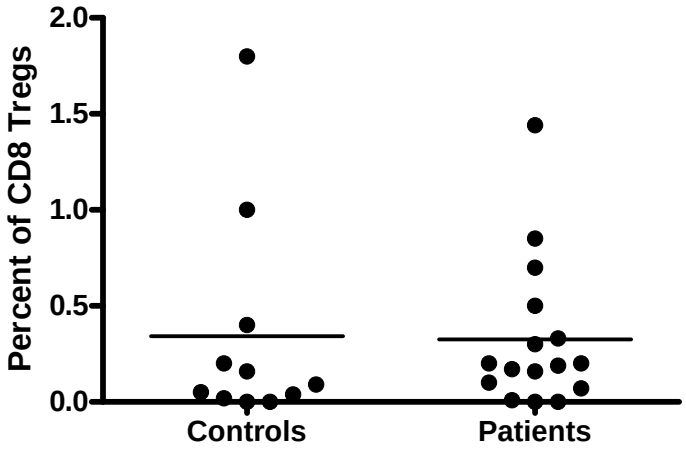
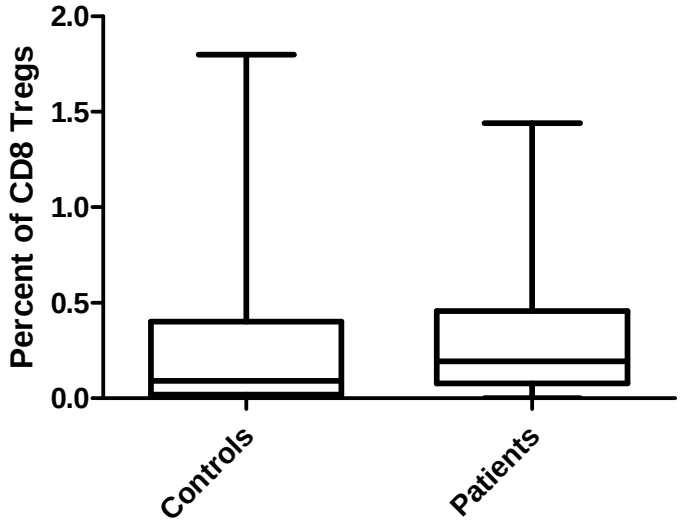


Figure 44 Graph to demonstrate the variance in percent of CD8 regulatory T cells expressed as a percentage of total CD8 positive lymphocytes in the peripheral blood of patient samples from patients with FL compared to healthy controls



6.4 Discussion

This study has investigated the percentage of regulatory T lymphocytes in the peripheral blood of patients with FL. It is commonly observed that immunological dysregulation is present in people with malignancy. This may be as a result of several factors including the impact of the tumour on the T lymphocytes causing dysfunction (Gorgun G et al. 2005) or the regulation of the immune system by regulatory T lymphocytes. The presence of increased regulatory T lymphocytes has been correlated to worse outcome in numerous epithelial malignancies (Curiel TJ et al. 2004; Loddenkemper C et al. 2006; Fu J et al. 2007). The work outlined in Chapter Five of this thesis also demonstrated an association between the perifollicular location of FOXP3 positive cells and improved outcome. In contrast, recent work has observed the association between shorter OS and the presence of intrafollicular FOXP3 positive cells (Kelley T et al. 2007). All of these studies have been performed on lymph node biopsies of FL, to date no data had been produced investigating the levels of regulatory T lymphocytes in the peripheral blood of patients with FL. In CLL, the presence of increased levels of CD4 positive regulatory T cells in the peripheral blood of patients with untreated or progressive disease as compared to peripheral blood of controls was observed. These regulatory T cells were functional and their numbers decreased after treatment with fludarabine (Beyer M et al. 2005).

This work has demonstrated that there is no significant increase in the percent of CD4 positive regulatory T lymphocytes in the peripheral blood of patients with FL compared to healthy controls. This is different to the observations in epithelial malignancies however, as previously discussed, the differences between haematological malignancies and epithelial are wide. The relationship to outcome in patients with haematological malignancies, particularly FL, is much less clear. Previous work has demonstrated the presence of functional Tregs in B-Non Hodgkin's Lymphomas (Carreras J et al. 2006; Yang ZZ et al. 2006) and the expression of FOXP3 has been shown to correlate with improved outcome in FL (Carreras J et al. 2006, Lee AM et al. 2006). Therefore, it is not surprising that the levels of regulatory T lymphocytes in the peripheral blood of patients with FL should not be in keeping with that observed in patients with epithelial malignancies. In addition, previous work has used CD4+CD25^{high} to identify Tregs, a method which

will include some effector T lymphocytes. More recently, FOXP3 has also been used to indicate the presence of regulatory T lymphocytes. It has been demonstrated that T lymphocytes which are not regulatory in nature can transiently express FOXP3 (Gavin MA et al. 2006; Wang J et al. 2007). The inclusion in this study of CD127, which has been demonstrated to be down-regulated on FOXP3 expressing cells which exhibit suppressive activity (Liu W et al. 2006; Seddiki N et al. 2006), has improved the accuracy of detecting regulatory T lymphocytes. This was not the case in many previous studies which may account for some of the differences observed. In addition, the patients selected were at varying points within their disease and some may have received treatment which may have affected the results. Although there is no increase in the percent of CD4 positive regulatory cells in the peripheral blood of patients with FL as compared to controls, there was a statistically significant increase in variability in the patient group. This work has also confirmed the variability of CD25 expression on regulatory T cells.

The levels of CD8 regulatory T cells in patients with malignant tumours have not been investigated. This work has demonstrated that CD8 positive regulatory cells comprise only a small percent of the normal CD8 T lymphocyte compartment with a mean of 0.34% in healthy controls. There is no increase of CD8 positive regulatory cells in the peripheral blood of patients with FL as compared to healthy controls. CD8 positive regulatory T cells are not identified in lymph node biopsies of FL using double immunofluorescence for FOXP3 and CD8 (data not shown).

In addition, a decrease in the percent of CD3 positive cells was observed in the peripheral blood of patients with FL as compared to controls. This has been previously observed in patients with stomach cancer and was related to advanced stage of disease (Hong WS et al. 1995). These results suggest that host immunity is impaired with advancing disease. Alternatively this could be a reflection of treatment although, treated patients were observed to have higher CD3 counts compared to patients treated by expectant management only.

CHAPTER SEVEN:

The likelihood of rapid transformation of FL to DLBCL can be predicted from the immune microenvironment in diagnostic FL lymph node biopsies

7.1 Introduction

Further to the previous data in FL we wished to determine if the immune microenvironment in diagnostic lymph node biopsies from patients with FL could predict the likelihood of rapid transformation of FL to DLBCL. Transformation is a relatively common and catastrophic clinical event. There are few reliable methods to predict likelihood of transformation, although histological grading is prognostic for outcome (Martin AR et al. 1995; Barlett NL et al. 1994) it is poorly reproducible (Metter GE et al. 1985). The aim of this work was to study the non-malignant immune infiltrate in diagnostic biopsies of FL and explore if the likelihood of rapid transformation could be determined from the composition of the immune infiltrate at diagnosis.

7.2 Materials and Methods

7.2.1 Patient samples

Patients diagnosed with FL at St Bartholomews' Hospital who had initial diagnostic paraffin embedded lymph nodes blocks and who subsequently transformed to DLBCL were eligible for this study. Twenty six samples were identified from patients who transformed from FL to DLBCL in less than 3 years from initial diagnosis and twenty five samples were identified from patients who transformed after eight years from initial diagnosis. Ethical approval for this study was obtained from the local regional ethics board.

7.2.2 TMA construction

Full sized haematoxylin and eosin stained sections were marked to indicate appropriate areas and 1mm cores taken in triplicate from the paraffin embedded tissue using a manual arrayer (Beecher Scientific, Sun Prairie, WI, USA). Six cores of control lymphoid tissue (appendix and tonsil) were also included on all TMAs.

7.2.3 Immunocytochemistry

The panel of antibodies applied to the TMA for analysis is listed in **Table 16**. TMA blocks were cut to a thickness of 4μm and applied to 3-aminopropyltrioxysilane-coated slides, dewaxed and blocked in hydrogen peroxide/methanol solution. Antigen retrieval was performed by pressure cooking in citrate except for FOXP3 where pressure cooking was performed in EDTA (Norton AJ, Jordan S, Yeomans P

1994). Sections were stained using the Vector Elite ABC kit (PK6100; Vector Laboratories, Peterborough, UK) or for FOXP3, CSA II tyramide amplification system (DAKO, Glostrup, Denmark) followed by diaminobenzidine chromogen (Biostat, Stockport, UK). Appropriate positive and negative controls were always used.

Table 16 Antibodies used for the investigation of the immune microenvironment

Test Antibody	**Clone**	**Source**	**Dilution**
CD4	NCL-CD4-368 clone 4B12	Novocastra, Newcastle, UK	1/100
CD8	C8/144B	Dako	1/50
CD68	KP1	Dako	1/4000
FOXP3	Ab236A/E7	Abcam, Cambridge, UK	1/40
CD21	NCL-CD21- 2G9	Novocastra, Newcastle, UK	1/50
CD23	NCL-CD23-1B12	Novocastra, Newcastle, UK	1/50

7.2.4 Immunocytochemistry analysis

The presence of adequate amounts of FL was confirmed using a routine diagnostic lymphoma panel composed of CD3, CD5, CD10, CD20, CD21, CD23, Bcl-2, Bcl-6, and Ki-67 (data not shown).

The TMAs were scored by myself and another independent histopathologist and consensus reached in all cases. The entire 1mm core was analysed at both low and high power in each case. Where a single core could not be scored a consensus of two cores for a case was accepted although this occurred in only 10% of the cores analysed. If two of the three cores were unassessable no scoring was attempted.

7.3 Results

7.3.1 Patient characteristics

Fifty one patients with diagnostic FL biopsies were eligible to be included within this study. The histology was reviewed in all cases. The characteristics of the patient population are listed in **Table 17**. Patients who underwent transformation of FL to DLBCL within three years of diagnosis, the rapidly transforming group (n=26), had a median time to transformation of 1.14 years (range 0-2.99). Patients who transformed eight years or more after diagnosis, the slow transforming group (n=25), had a median time to transformation of 19.02 years (range 8.15-34.02). The members of the rapidly transforming were older than the slowly transforming, with median ages of 61 years and 47 years respectively. This was not statistically significant (p=0.18).

Table 17 Demographic and Clinical Characteristics of Patients from the Rapidly Transforming and Slow Transforming Groups

	Rapidly transforming group	**Slow transforming group**
Total no. of patients (n=51)	26	25
Sex		
Male	18	15
Female	8	10
Time to transformation (years)		
Median	1.14	19.02
Range	0 – 2.99	8.15 – 34.02
Age at diagnosis (years)		
Median	61.04	47
Range	30.8 - 72.6	30.5 - 65
Grade	Grade 1 = 13 Grade 2 = 11 Grade 3a = 2	Grade 1 = 14 Grade 2 = 8 Grade 3a = 3
Stage	2 unknown	
I	0	1
II	5	6
III	8	6
IV	11	12
Number of repeat biopsies		15 cases: 1-6 relapses, 1-7 biopsies 6 cases: no relapse, no biopsies 1 case: no relapse, 3 biopsies 3 cases: 1 relapse, no biopsies

7.3.2 Analysis of TMA

For each of the antibodies studied, two (for a maximum of 10% of the patients' samples over all antibodies studied) or three cores were available for analysis from 24 to 25 of the rapidly transforming group and 21 to 25 of the slow transforming group. Scoring was performed as described previously based on location with respect to the neoplastic follicle and number of cells with categories as follows: less than five cells/hpf, five to 10 cells/hpf, 10-15 cells/hpf, 15-30 cells/hpf, and more than 30 cells/hpf. The analysis of these categories did not reveal any additional correlation with time to transformation and consequently the categories of less than five cells/hpf and more than five cells/hpf were used except for CD68; where the value less than 15 cells/hpf and more than 15 cells/hpf were substituted to correlate with the work by Farinha et al. (Farinha P et al. 2005).

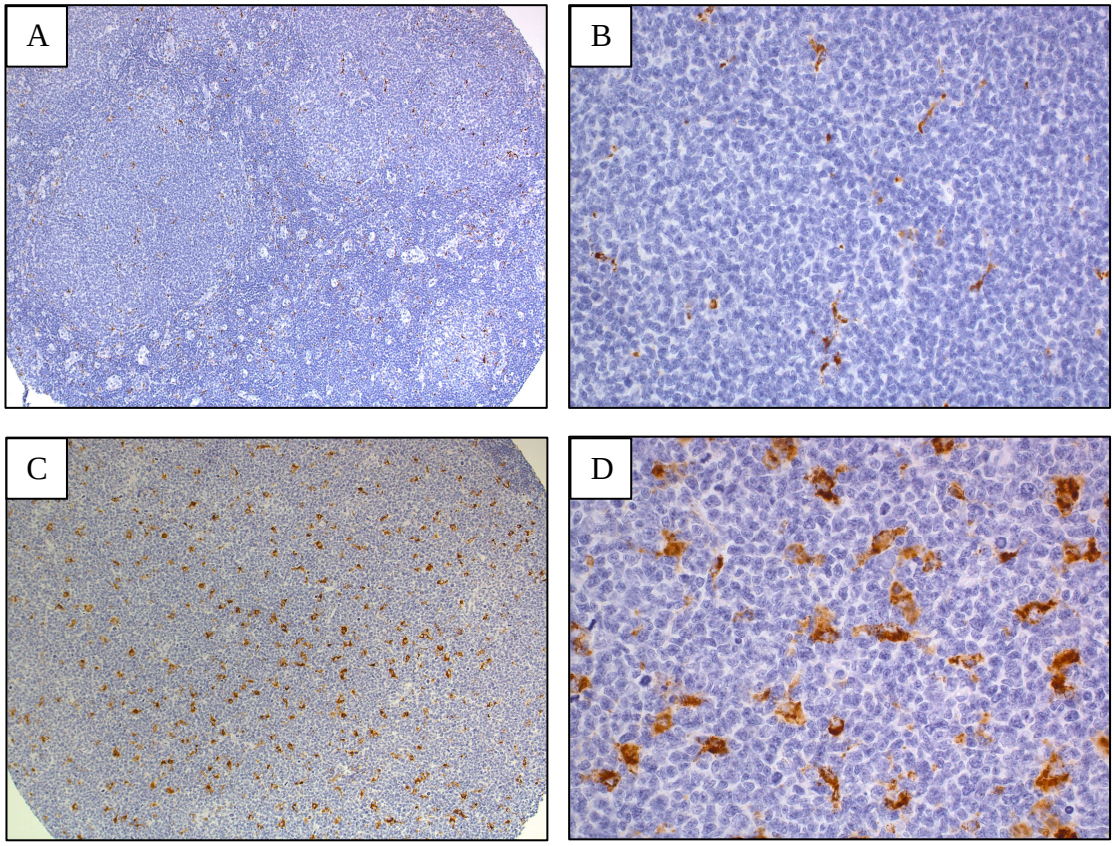
7.3.3 CD68 positive cell numbers are not significantly different between rapidly transforming and slow transforming groups

CD68 expression was assessable in 48 of 51 (94%) total patients; two of the unassessable patients were from the slowly transforming group and one of the patients was from the rapidly transforming group. CD68 positive cells were observed at levels of >15/hpf in 23 of 48 (48%) patients; 15 of 25 (65%) rapidly transforming group and 8/23 (35%) of biopsies from the slowly transforming group ($p=0.08$; **Table 18**) (**Figure 45**).

Table 18 Number of CD68 positive cells in diagnostic lymph node biopsies

	Number of assessable samples		<15 cells/hpf		>15 cells/hpf	
Antigen	Rapid transforming group	Slow transforming group	Rapid transforming group	Slow transforming group	Rapid transforming group	Slow transforming group
CD68	25	23	10/25 40%	15/23 65%	15/25 60%	8/23 35%
P			0.08			

Figure 45 Immunocytochemistry for CD68 on diagnostic FL samples, A) <15/hpf positive cells OM x10, B) <15/hpf positive cells OM x40, C) >15/hpf positive cells OM x10, D) >15/hpf positive cells OM x40



CD4 expression was assessable in 49 of 51 (96%) total patients; the two unassessable patients were both from the rapidly transforming group. The absence or presence of CD4 positive cells did not correlate with time to transformation. All patient samples analysed contained CD8 positive cells. FOXP3 expression was assessable in 48 of 51 (94%) of patients. Two of the patients whose samples were not assessable were from the rapidly transforming group and the other patient was from the slowly transforming group. The presence or absence of FOXP3 positive cells did not correlate with time to transformation (**Table 19**).

Table 19 Expression of immune cell antigens in diagnostic lymph node biopsies

	Number of assessable Samples		<5 cell/hpf		>5 cells/hpf	
Antigen	Rapid transforming group	Slow transforming group	Rapid transforming group	Slow transforming group	Rapid transforming group	Slow transforming group
CD4	24	24	2/24 8%	4/24 16%	22/24 92%	20/24 84%
P	0.4					
CD8	25	21	0/25 0%	0/21 0%	25/25 100%	21/21 100%
FOXP3	24	24	4/24 16%	7/24 29%	20/24 84%	17/24 71%
P	0.24					

7.3.4 Location of CD4 positive cells correlates with time to transformation

Patients from the rapidly transforming group had CD4 positive cells at a higher incidence within follicles (intrafollicular) than patients in the slow transforming group. Patients from the slowly transforming group contained FOXP3 positive cells in a predominantly intrafollicular location as compared to the patients in the rapidly transforming group. The location of CD8 positive cells did not differ significantly between the groups (**Table 20**).

Table 20 Location of immune cell antigens in diagnostic lymph nodes

	Number of assessable samples		Intrafollicular		Predominantly perifollicular	
Antigen	Rapid transforming group	Slow transforming group	Rapid transforming group	Slow transforming group	Rapid transforming group	Slow transforming group
CD4	24	24	15/24 63%	7/24 29%	6/24 25%	14/24 58%
P	0.01					
CD8	25	23	14/25 56%	12/23 51%	11/25 44%	11/23 48%
P	0.2					
FOXP3	24	24	8/24 33%	13/24 54%	12/24 50%	4/24 17%
P	0.04					

7.4 Discussion

This work highlights the importance of the immune microenvironment in outcome of haematological malignancy. The transformation of FL to DLBCL is a common event (Horning S & Rosenberg S 1984) which is clinically catastrophic for most patients (Acker B et al. 1983; Gallagher CJ et al. 1986). The biological mechanisms which underlie transformation are still unclear with numerous proposed molecular mechanisms including mutation of *TP53* (Lo Coco F et al. 1993; Sander CA et al. 1993; Davies AJ et al. 2005), cytogenetic abnormalities (Goff LK et al. 2000; Hough RE et al. 2001), amplifications of *c-REL* (Goff LK et al. 2000), deletions of *CDKN2A* and *CDKN2B* (Elenitoba-Johnson KS et al. 1998), mutations of *Bcl-2* (Matolcsy A et al. 1996) and mutations of *Bcl-6* (Lossos T, Levy R 2000). Each of these has been identified in small numbers of patients, however if treatments and survival for patients who undergo transformation are to be improved further insight into the biological mechanisms is required. Previous work has identified the importance of the immune microenvironment in outcome of FL both at the level of gene expression (Dave SS et al. 2004) and protein expression within biopsies of tumour (Farinha P et al. 2005; Carreras J et al. 2006; Lee AM et al. 2006). This work has examined the immune microenvironment in diagnostic FL lymph node biopsies and evaluated its prognostic significance with respect to time to transformation.

There was a suggestion that increased numbers of CD68 positive macrophages, defined according to the criteria used by Farinha et al., were more commonly found in the diagnostic FL lymph nodes of patients who underwent rapid transformation to DLBCL with a trend towards statistical significance ($p=0.08$) These findings are in keeping with the hypothesis that the lymphoma-associated macrophage content is an independent predictor of survival in FL but this will require further validation using a larger sample size. The data produced by Farinha et al. is not directly comparable with this study as the endpoints used were progression-free survival and overall survival (Farinha P et al. 2005) whereas this work used time to transformation as the endpoint. However, these results are indicative that high numbers of macrophages are important in outcome of FL either time to transformation or survival. Moreover the use of immunocytochemistry and defined criteria for analysis of this biomarker would enable widespread utilisation and may be of prognostic help to clinicians when determining treatment. The role which macrophages play in this setting is not

fully understood. Macrophages are attracted into the tumour microenvironment by chemokines produced by the tumour cells. Dependent upon the environment macrophages can be activated either into an M1 form which is activated in response to microbial products and cytokines particularly interferon-γ (Adams DO, Hamilton TA 1984) or an M2 (alternatively activated) form (Stein M et al. 1992). Conversion into the M2 form is induced by IL-4 (Stein M et al. 1992), IL-13 and IL-10 (Goerdt S; Orfanos CE 1999). These two polarised macrophage types differ in receptor expression, cytokine production and function. The alternatively activated macrophages are able to endocytose mannosylated ligands and demonstrate enhanced expression of MHC class II molecules. They also produced less pro-inflammatory cytokines (Stein M et al. 1992). In addition, phagocytosis of immune complexes and production of superoxide were inhibited by exposure of macrophages to IL-4. This effect was reversed by exposure to IFN-γ (Becker S, Daniel EG 1990). Generally the M1 macrophages produced plentiful pro-inflammatory cytokines and mount effective cytotoxic responses against micro-organisms and tumours whereas the M2 macrophages are responsible for remodelling and healing. Activated macrophages appear to have several mechanisms of action. They express anti-inflammatory cytokines e.g. IL-10 (Fenton MJ, Buras JA, Donnelly RP 1992) and they lack expression of proinflammatory cytokines e.g. IL-1, IL-6, IL-12, macrophage inflammatory protein and TNFα (Cheung DL et al. 1990; Standiford TJ et al. 1993; Bonder CS et al. 1998).

The presence of increased numbers of macrophages correlating with worse outcome is unsurprising on consideration of the functions the M2 group perform. The presence of these cells would reduce any inflammatory reaction against the tumour cells as well as producing angiogenic factors which could increase the vascularisation of the tumour. Immune manipulation of the host may prevent recruitment of the M2 cells into the tumour microenvironment allowing a host immune response to destroy the tumour.

A study performed by Glas et al. has produced similar results for the number and location of CD4 positive T lymphocytes. Patients who transformed rapidly from FL to DLBCL (within three years from diagnosis) demonstrated a predominantly perifollicular pattern in only 3/24 cases and a predominantly intrafollicular pattern in

10/24 cases. Patients who did not transform within seven years of diagnosis, demonstrated a predominantly perifollicular pattern in 20/32 cases and a predominantly intrafollicular pattern in 2/32 cases (Glas AM et al. 2007) The data presented here is also similar to the work of Glas et al. as both did not find any association with the number of CD8 positive T cells. The hypothesis generated by their gene expression data indicated that gene expression patterns in rapidly transforming FL were more comparable to those in non-malignant follicular hyperplasia, while the non transforming group demonstrated overall down-regulation of immune related genes and resembled non-activated lymphoid tissue (Glas AM et al. 2007). This would certain fit with the data presented here. In addition, the presence of FoxP3 positive cells being present intrafollicularly in 13/24 cases from the slow transforming group could be explained by the FoxP3 positive cells acting to suppress the activation of the lymphocytes and so maintain an immunologically inactive state (Glas AM et al. 2007).

The findings relating to CD4 lymphocytes support the model proposed by Glas et al. where FL resembling non-malignant hyperplasia was associated with rapid transformation (Glas AM et al. 2007). It also implies that the presence of FoxP3 positive cells within the neoplastic follicle can improve prognosis by exerting a suppressive effect on the non-malignant immune infiltrate so attaining a non-activated lymphoid tissue phenotype which is associated with a non-transforming FL. The presence of high numbers of macrophages correlating with rapid transformation is in keeping with the data produced by Farinha et al. as transformation can be a catastrophic event which results in death at a much earlier timepoint than if transformation did not occur (Farinha P et al. 2004).

The results also indicate that the nature of the non-immune infiltrate at diagnosis is important in determining prognosis as well as providing biomarkers which may help identify patients who may rapidly transform and so need close observation and possibly more aggressive therapy. In addition, these markers can be assessed routinely at the time of diagnosis with little increased work to the reporting histopathologist.

CHAPTER EIGHT:

The impact of the immune microenvironment on transformation of FL to DLBCL

8.1 Introduction

As previously outlined, the immune microenvironment is extremely important in the natural history of FL. Transformation of FL to DLBCL is a relatively common and clinically catastrophic event. The aim of this work was to investigate the impact of the microenvironment on the process of transformation. This was achieved by analysis of TMAs composed of 1mm triplicate cores of biopsies from paired pre-transformation and transformed samples from patients who presented with FL. The samples were analysed to determine the composition of the immune microenvironment in the pre-transformation biopsies and whether this changed after transformation had occurred.

8.2 Patients and methods

8.2.1 Patient samples

A cohort of 31 patients with diagnostic paraffin embedded FL biopsies who subsequently transformed to DLBCL was identified and the immune microenvironment analysed. In addition a further cohort of 31 patients with initial diagnostic paraffin-embedded de novo DLBCL biopsy samples was identified from the archives of the Pathology department of St Bartholomew's Hospital. The immune microenvironment was analysed in these samples and compared with the immune microenvironment in the transformed samples.

A cohort of 34 patients diagnosed with FL who subsequently transformed to DLBCL with at least one pre-transformation and post-transformation paraffin-embedded biopsy samples was identified from the archives of the Pathology department of St Bartholomew's Hospital as described previously. Ethical approval for this study was obtained from the local regional ethics board. Tissue microarrays (TMAs) were constructed from these samples (transformed TMA).

8.2.2 TMA construction

Full sized haematoxylin and eosin stained sections were marked to indicate appropriate areas and 1mm cores taken in triplicate from the paraffin embedded tissue using a manual arrayer (Beecher Scientific, Sun Prairie, WI, USA). All cores from a single patient were included on the same TMA. Six cores of control lymphoid

tissue (appendix and tonsil) were also included on all TMAs.

8.2.3 Immunocytochemistry

The panel of antibodies and dilutions applied to the TMAs for analysis are listed in **Table 21**. TMA blocks were cut to a thickness of 4μm and applied to 3-aminopropyltrioxysilane-coated slides, dewaxed and blocked in hydrogen peroxide/methanol solution. Antigen retrieval was performed by pressure cooking in citrate except for FOXP3 where pressure cooking was performed in EDTA (Norton AJ, Jordan S, Yeomans P 1994). Sections were stained using the Vector Elite ABC kit (PK6100; Vector Laboratories, Peterborough, UK) or for FOXP3, CSA II tyramide amplification system (DAKO, Glostrup, Denmark) followed by diaminobenzidine chromogen (Biostat, Stockport, UK). Appropriate positive and negative controls were always used.

Table 21 Antibodies used for the investigation of the immune microenvironment

Test Antibody	**Clone**	**Source**	**Dilution**
CD4	NCL-CD4-368 clone 4B12	Novocastra, Newcastle, UK	1/100
CD8	C8/144B	Dako	1/50
CD68	KP1	Dako	1/4000
FOXP3	Ab236A/E7	Abcam, Cambridge, UK	1/40
CD21	NCL-CD21- 2G9	Novocastra, Newcastle, UK	1/50
CD23	NCL-CD23-1B12	Novocastra, Newcastle, UK	1/50

8.2.4 Immunocytochemistry analysis

The presence of adequate amounts of FL or DLBCL was confirmed using a routine diagnostic lymphoma panel composed of CD3, CD5, CD10, CD20, CD21, CD23, Bcl-2, Bcl-6, and Ki-67 (data not shown).

The TMAs were scored by myself and a second independent histopathologist who were blinded to the layout and consensus reached in all cases. The entire 1mm core was analysed at both low and high power in each case. Where a single core could not be scored a consensus of two cores for a case was accepted although this occurred in only 10% of the cores analysed. If two of the three cores were unassessable no scoring was attempted.

Staining was evaluated based on the number and location relative to the neoplastic follicle of positively stained cells in the follicular lymphoma biopsies. Numerical evaluation was divided into the following categories; less than five cells/hpf, five to 10 cells/hpf, 10-15 cells/hpf, 15-30 cells/hpf, and more than 30 cells/hpf.

8.3 Results

8.3.1 Immune microenvironment in transformed TMA

8.3.1.1 Patient characteristics

Biopsies from 34 FL patients who transformed to DLBCL with at least one pre-transformation and post-transformation paraffin-embedded biopsy samples available were identified. The clinical characteristics are detailed in **Table 22**.

Table 22 Patient characteristics of patients in transformed TMA

	FL (n=34)
Median age at diagnosis	52 years (range 31-71 years)
Male to female	Male: 17 Female: 17
Stage	Unknown = 5 Stage 1 = 1 Stage 2 = 4 Stage 3 = 10 Stage 4 = 14
Grade	Grade 1 = 16 Grade 2 = 18 Grade 3 = 0
Median time to transformation from diagnosis (years)	3.3 years (range 0-15 years)
Median OS from diagnosis (years) (to death or last follow-up)	7.1 years (range 0.5 -17.3 years) 8 patients still alive
Median time from transformation to death/last follow up (years)	2.4 years (range 0.17 – 12.7 years)

8.3.1.2 CD4 positive lymphocytes

Twenty two paired samples were available for analysis from a possible 34 due to core loss. The number of CD4 positive cells was generally high in all samples. The number of CD4 positive lymphocytes either increased or remained high on transformation in 15/22 cases (68%). The numbers decreased or remained low on transformation in 7/22 cases (32%) (p=0.003). There was no significant difference in overall survival or survival from transformation based on changes of CD4 positive lymphocytes on transformation.

8.3.1.3 CD8 positive lymphocytes

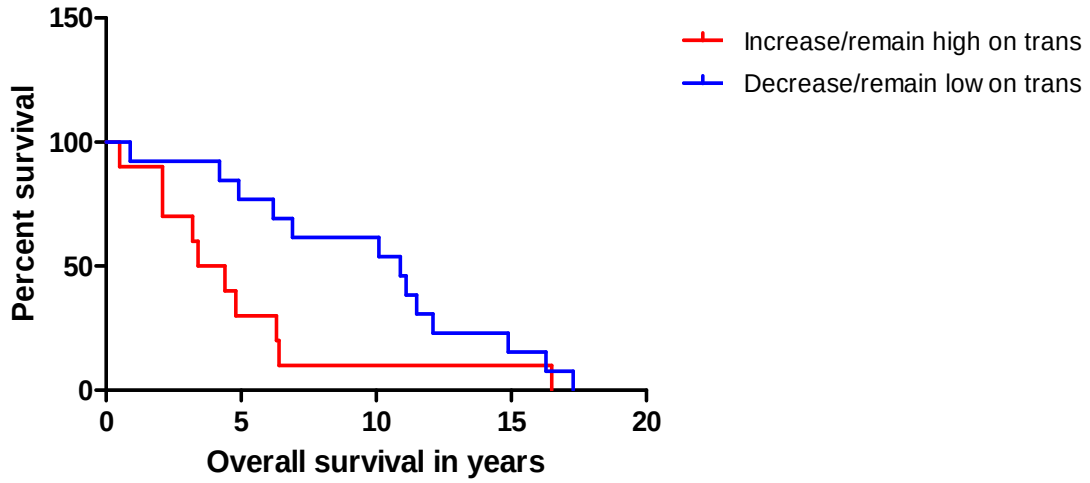
Twenty two paired samples were available for analysis from a possible 34 due to core loss and the number of CD8 positive cells was high in all FL samples. The

number of CD8 positive lymphocytes increased or remained high in 17/22 cases (77%). The numbers decreased in 5/22 cases (23%)(p=<0.05). There was no significant difference in overall survival or survival from transformation based on changes of CD8 positive lymphocytes on transformation.

8.3.1.4 FOXP3 positive cells

Twenty three paired samples were available for analysis from a possible 34 due to core loss. The number of FOXP3 positive cells fell or remained low on transformation in 13/23 cases (56%). The numbers of FOXP3 positive cells increased marginally (from 5-10 cells/hpf to 10-15 cells/hpf) in 8/23 cases (35%). In two paired samples the number of FOXP3 positive cells remained high (9%)(p=0.23). There was a statistically significant difference in OS in patients based on the change in FOXP3 numbers on transformation from FL to DLBCL. Patients with biopsies in which the number of FOXP3 positive cells decreased on transformation survived for a median of 10.9 years from initial diagnosis of FL compared to patients whose numbers of FOXP3 cells increased on transformation who survived a median of 3.9 years from initial diagnosis (p=0.01) (**Figure 46**)

Figure 46 Kaplan-Meier estimates of overall survival of patients with transformed FL by change in number of FOXP3 positive cells (p=0.01). Blue indicates samples in which the number of FOXP3 positive cells decreased or remained low in both the FL and transformed DLBCL samples. Red indicates those samples where the numbers of FOXP3 positive cells increased or remained high in the transformed DLBCL sample.



8.3.1.5 CD68 positive macrophages

Twenty four paired samples were available for analysis from a possible 34 due to core loss. The number of CD68 positive macrophages remained high or increased in 23/24 cases (96%). In a single case a marginal decrease was observed (p=<0.05).

8.3.2 Assessment of immune microenvironment in de novo DLBCL

8.3.2.1 Patient characteristics

Twenty two patients with diagnostic *de novo* DLBCL paraffin embedded biopsies were available for analysis from 31 potential patients. The patient characteristics are described in **Table 23**. Twelve patients are still alive, all deceased patients died as a result of their disease.

Table 23 Clinical features of patients with *de novo* DLBCL

	De novo DLBCL (n=22)
Median age at diagnosis	50 (range 18-83 years)
Male: female	9 male 13 female
Stage at diagnosis	Stage 1=3 Stage 2=1 Stage 3=6 Stage 4=8 Stage 1E=1 Stage IIE=3
Median survival (years)	4 years (range 0.5–11.4 years) 12 patients still alive
International Prognostic Index (IPI)	Unknown = 3 Low risk = 13 Low-intermediate = 2 High intermediate = 3 High risk = 1
Germinal centre type versus Non germinal centre	GC type = 9 Non GC type = 13

8.3.2.2 CD4 positive cells

Of the twenty two evaluable cases, 15 biopsies were assessable for number of CD4 positive cells. Three of the 15 cases (20%) contained <5 cells/hpf and 12 of the 15 cases (80%) contained >5 cells/hpf.

8.3.2.3 CD8 positive cells

Sixteen of twenty two evaluable cases were suitable for expression of CD8 positive cells; only one case (6%) contained <5 positive cells/hpf. The remaining 15 cases (94%) contained >5 positive cells/hpf.

8.3.2.4 CD68 positive macrophages

Fifteen of twenty two available cases were assessable for expression of CD68 positive macrophages. Only one case (7%) contained <5 positive cells/hpf. The remaining 14 cases (93%) contained >5 positive cells. Seven of 15 cases (47%) contained >15 positive cells/hpf and 8 cases (53%) contained <15 positive cells/hpf

8.3.2.5 FOXP3 positive cells

Seventeen cases of twenty two were suitable for analysis of FOXP3 positive cells. Four cases (24%) contained <5 positive cells/ hpf; 13 cases (76%) contained >5 positive cells/ hpf.

Table 24 Cumulative results for the immune microenvironment composition in de novo DLBCL samples

	CD4 (n=15)	**CD8 (n=16)**	**CD68 (n=15)**	**FOXP3 n=17)**
<5 cells/hpf	3 (20%)	1 (6%)	1 (7%)	4 (24%)
5-10 cells/hpf	0 (0%)	1 (6%)	4 (27%)	5 (29%)
10-15 cells/hpf	1 (7%)	1 (6%)	3 (20%)	2 (12%)
15-20 cells/hpf	0 (0%)	0 (0%)	0 (0%)	2 (12%)
>30 cells/hpf	11 (73%)	13 (81%)	7 (47%)	4 (24%)

8.3.2.6 The immune microenvironment does not influence outcome in de novo DLBCL

The group of twenty two patients was then divided according to their OS with the median OS of the whole group (4 years) used to split the patients.The median OS of the patient group below the median was 1 year and the median OS of the patient group above the median was 7.3 years which was statistically significant (p=0.02). The patient group with a worse OS had a higher median age at diagnosis (54 years) than the patient group with an improved OS (49 years) however this was not statistically significant (p=0.6).

Table 25 Patient characteristics of two groups of patients with de novo DLBCL divided on the basis of OS

	Survival < median (n=11)	**Survival >median (n=11)**
Median age at diagnosis (years)	54 years	49 years
Male/ female ratio	Male = 2 Female =9	Male = 7 Female = 4
Median OS (years)	1 year 1 patient still alive	7.3 years All alive at the present time
GC type versus non GC type	GC = 4 Non GC = 7	GC = 5 Non GC = 6
Stage	Stage 1 = 0 Stage 2 = 0 Stage 3 = 3 Stage 4= 6 Unknown = 2	Stage 1 = 3 Stage 2 = 1 Stage 3 = 3 Stage 4 = 2 Unknown = 2
IPI	Low = 3 Low intermediate = 2 High intermediate = 2 High = 1 Unknown = 3	Low = 10 Low intermediate = 0 High intermediate = 1 High = 0 Unknown = 0

All four markers; CD4, CD8, CD68 and FOXP3 were analysed for their impact on outcome in de novo DLBCL. There were no significant differences between the groups for any of the markers. It was observed however that all samples from patients in the longer survival group contained >30 CD4 positive cells per hpf however this was not statistically significant. The only prognostic marker appeared to be the IPI, all patients in the long survivor group were classified in the low IPI group except for one patient classified as high –intermediate. The patients who died quickly from their disease were in varying IPI groups; three were in the high and high intermediate groups and five were in the low and low intermediate groups. The IPI of three patients in this survival group were unknown.

8.3.3 Immune microenvironment in diagnostic FL samples of patients who subsequently transform to DLBCL

8.3.3.1 Patient characteristics

The patient characteristics from thirty one patients diagnosed with FL who subsequently transformed to DLBCL and whose initial diagnostic lymph nodes were available are listed in **Table 26**.

Table 26 Patient characteristics for patients diagnosed with FL who subsequently transformed to DLBCL

	Diagnostic FL who transform (n=31)
Median age at diagnosis (years)	56 (range 31 – 79)
Male to female ratio	Male = 22 Female = 9
Grade	Grade 1 = 16 Grade 2 = 13 Grade 3 = 2
Stage	Unknown = 3 Stage 1 = 1 Stage 2 = 4 Stage 3 =8 Stage 4= 14
Median time to transformation from diagnosis of FL (years)	2 years (range 0.1 – 13.6 years)
Median OS from diagnosis of FL (years)	4 years (range 0.5 - 26.1 years)

8.3.3.2 CD4 positive lymphocytes

Twenty nine cases from thirty one possible were evaluable for analysis of CD4 positive lymphocytes. Twenty four cases (83%) contained more than 5 positive cells/hpf and 5 cases (17%) contained less than 5 positive cells/hpf. Of the twenty four cases thirteen of these (45%) contained CD4 positive cells in a predominantly perifollicular pattern and 11 cases (38%) the cells were evenly distributed both within and around the neoplastic follicle.

8.3.3.3 CD8 positive lymphocytes

Thirty cases of thirty one available were analysed for expression of CD8 positive lymphocytes. All thirty cases contained >5 positive cells/hpf. Twenty three cases (73%) contained CD8 positive cells in a predominantly perifollicular pattern and in 7

cases (27%) the cells were evenly distributed both within and around the neoplastic follicle.

8.3.3.4 CD68 positive macrophages

Thirty cases were available for analysis of expression of CD68 positive macrophages. Twenty seven of thirty cases contained >5 positive cells/hpf (90%) and three cases contained <5 positive cells/hpf (10%). Fourteen of thirty cases (47%) contained >15 positive cells/hpf; sixteen cases (53%) contained <15 positive cells/hpf.

8.3.3.5 FOXP3 positive cells

Of the twenty nine evaluable cases twenty four cases (83%) contained >5 positive cells/hpf and five cases (17%) contained <5 positive cells/hpf. Of the twenty four cases; fifteen cases (52%) contained cells in a predominantly perifollicular location; 1 case (3%) contained cells in a predominantly intrafollicular location. The cells were evenly distributed in 8 cases (28%).

Table 27 Cumulative results for the immune microenvironment composition in diagnostic FL samples from patients who transform to DLBCL

	CD4 (n=29)	**CD8 (n=30)**	**CD68 (n=30)**	**FOXP3 (n=29)**
<5 cells/hpf	5 (17%)	0 (0%)	3 (10%)	5 (17%)
5-10 cells/hpf	1 (3%)	5 (17%)	12 (40%)	11 (38%)
10-15 cells/hpf	4 (14%)	4 (13%)	1 (3%)	6 (21%)
15-20 cells/hpf	4 (14%)	4 (13%)	3 (10%)	2 (7%)
>30 cells/hpf	15 (52%)	17 (57%)	11 (37%)	5 (17%)

8.3.3.6 The impact of the immune environment at diagnosis on outcome in patients with FL who transform to DLBCL

Thirty one patients were analysed; eleven patients had an OS beyond the median for the group (4 years) and were termed patients with 'good survival'. Twenty patients had an OS below the median for the group and were termed patients with 'poor survival'. These numbers are very small to assess the impact of the immune microenvironment at diagnosis on OS. The patient characteristics of these groups are outlined in **Table 28**. There was no statistically significant difference in median age at diagnosis between the two patient groups (61 years versus 52 years; p=0.4). There was no significant correlation with survival when the numbers of immune infiltrating cells were analysed for any of the populations. It is worth noting that the biopsies from the majority of patients with a survival above the median contained <15 macrophages per hpf (73%)(**Table 29**).

Table 28 Patient characteristics for two groups of patients with diagnostic samples who transform to DLBCL divided on the basis of median OS

	Patients who survived < median (poor survival) (n = 20)	**Patients who survived > median (good survival) (n = 11)**
Median age at diagnosis (years)	61 years (range 31 – 79)	52 years (range 31 – 71)
Male : female ratio	Male = 15 Female = 5	Male = 7 Female = 4
Median OS (years)	3 years (range 0.5 – 3.4)	8.1 years (range 4.8 – 26.1)
Grade	Grade 1 = 11 Grade 2 = 8 Grade 3 = 1	Grade 1 = 5 Grade 2 = 5 Grade 3 = 1
Stage	Unknown = 2 Stage 1 = 1 Stage 2 = 3 Stage 3 = 4 Stage 4 = 10	Unknown = 1 Stage 1 = 0 Stage 2 = 1 Stage 3 = 4 Stage 4 = 5

Table 29 Cumulative results for the number of cells present in the immune microenvironment in initial diagnostic samples from patients who transformed to DLBCL

	CD4 (n=29)		**CD8 (n=30)**		**CD68 (n=30)**		**FOXP3 (n=29)**	
Number of cells	<5/hpf	>5/hpf	<5/hpf	>5/hpf	<15/hpf	>15/hpf	<5/hpf	>5/hpf
Poor survival (n=18)	3 (17%)	15 (83%)	0 (0%)	19 (100%)	8 (40%)	11 (60%)	4 (22%)	14 (78%)
Good survival (n=11)	2 (18%)	9 (82%)	0 (0%)	11 (100%)	8 (73%)	3 (27%)	1 (9%)	10 (91%)
P value	P= 0.6		N/A		P= 0.1		P= 0.4	

8.3.3.7 Impact of location of immune microenvironment cells on OS

The location of infiltrating immune cells with regard to the neoplastic follicle was also assessed. The location of any of the assessed immune cell populations did not appear to impact on survival (**Table 30**).

Table 30 Cumulative results for the location of immune infiltrating cells with regard to the neoplastic follicle in the diagnostic FL biopsies of patients who subsequently transformed to DLBCL

	CD4 (n=29)				**CD8 (n=30)**				**CD68 (n=30)**				**FOXP3 (n=29)**			
Location of cells	<5/hpf	Perif	Intraf	Evenly	<5/hpf	Perif	Intraf	Evenly	<15/hpf	Perif	Intraf	Evenly	<5/hpf	Perif	Intraf	Evenly
Poor prognosis (n=20)	3 (15%)	10 (50%)	0 (0%)	5 (25%)	0 (0%)	13 (65%)	0 (0%)	6 (30%)	8 (40%)	5 (25%)	1 (5%)	5 (25%)	4 (20%)	9 (45%)	1 (5%)	4 (20%)
Good prognosis (n==11)	2 (18%)	3 (27%)	0 (0%)	6 (55%)	0 (0%)	9 (82%)	0 (0%)	2 (18%)	8 (72%)	2 (18%)	0 (0%)	1 (9%)	1 (9%)	6 (55%)	0 (0%)	4 (36%)
P value	P= 0.2				P=0.67				P =0.47				P =0.75			

Evenly = evenly distributed both within and around the neoplastic follicle

Perif = predominantly perifollicular in location

Intraf= predominantly intrafollicular in location

8.4 Discussion

Previous work in this thesis has demonstrated the importance of the immune microenvironment in the natural history of FL. Transformation to DLBCL is a catastrophic and relatively common clinical event but to date examination of the microenvironment on transformation of FL to DLBCL has been limited. Carreras et al. demonstrated a reduction in numbers of FOXP3 positive cells on transformation of FL to DLBCL (Carreras J et al. 2006). My work has demonstrated that the number of cells positive for FOXP3 decreased or remained low in 56% of cases which transformed from FL to DLBCL however the number of FOXP3 positive cells increased or remained high in 45% of patients. This was not statistically significant and differs to the Carreras data. This may be a reflection of the differing methods used to count the FOXP3 positive cells as well as sample variance, Carreras et al. used computer-analysis of full sections, studied more patients with a mixed and diffuse histology and defined location differently (Carreras J et al. 2006). There was a statistically significant difference in OS in patients based on the change in FOXP3 numbers on transformation from FL to DLBCL. Patients with biopsies in which the number of FOXP3 positive cells decreased on transformation survived for a median of 10.9 years from initial diagnosis of FL compared to patients whose numbers of FOXP3 cells increased on transformation who survived a median of 3.9 years from initial diagnosis ($p=0.01$) This may represent the impact of the immune system being able to regulate the tumour growth without regulatory cells inhibiting the anti-tumour response. The number of CD68 positive macrophages was high in all samples of FL prior to transformation except one and remained so on transformation to DLBCL. This would be in keeping with the observations of Farinha et al. who using immunohistochemistry for CD68 observed a short survival correlated with >15 positive staining cells/hpf in diagnostic biopsies (Farinha P et al. 2005). Transformation of FL to DLBCL is a clinically catastrophic event associated with shortened OS which would correlate with the findings of Farinha et al. (Farinha P et al. 2005). For both CD4 and CD8 lymphocytes the majority of cases (68% and 77% of cases respectively) demonstrated an increase in numbers or remained high on transformation. This could represent an attempt by the immune system to regulate the now rapidly growing tumour. The morphological changes in the tumour might be accompanied by development of tumour antigens to which the immune system could respond and try to destroy the tumour. There was no significant difference in OS or

survival from transformation based on changes in the numbers of CD4 or CD8 lymphocytes on transformation.

Gene expression profiling has previously identified a subtype of de novo DLBCL including one delineated by host inflammatory response. This subtype was characterised by having higher numbers of infiltrating T and NK cells. The immune microenvironment did not impact on overall survival in patients with de novo DLBCL in my work. Although there was no statistically significant impact on OS of any subset of immune cells it was observed however that all samples from patients in the longer survival group contained >30 CD4 positive cells per hpf. This would be in keeping with previous work where the presence of high numbers of CD4 positive T lymphocytes was associated with significantly longer 5 year failure-free survival as well as being an independent predictor of relapse free and overall survival (Ansell SM et al. 2001). This was also demonstrated in a study using flow cytometry where cases with high numbers of T lymphocytes (>20%) and a CD4:CD8 ratio of >2.0 demonstrated significantly improved overall survival (Xu Y et al. 2001). The lack of impact on overall survival of FOXP3 positive cells in this work has also been demonstrated in another study (Hasselblom S et al. 2007). Eighty one percent of the de novo DLBCL cases contained high numbers of CD8 positive T lymphocytes and there was no correlation with OS which differed from previously published work. This work, although conflicting did demonstrate a correlation with OS; low numbers of CD8 positive T lymphocytes were associated with a poor outcome in one study (Lippman SM et al. 1990) however the presence of >15% of activated cytolytic T lymphocytes was strongly associated with failure to reach complete remission, with a poor progression-free and overall survival in a different study (Muris JJ et al. 2004). The study by Lippman et al did observe that most cases of DLBCL contained high numbers of CD8 lymphocytes, as we observed in this work and the lack of correlation with OS in our study may represent a lack of patient samples (n=22) as compared to the Lippman study (n=82) (Lippman SM et al. 1990). The study by Muris et al. used immunohistochemistry for CD3 and granzyme B and a software system to overlay the staining (Muris JJ et al. 2004). My work has used CD8 to identify cytolytic T lymphocytes and scoring was performed by histopathologists as well as being performed on a much smaller number of cases (n=22 versus n=70) which could account for the differences. The only predictor of outcome in this work

appeared to be the IPI with all patients who survived longer than the median survival being in the low IPI group apart from one in the high intermediate group.

The number of immune cells in the diagnostic FL lymph node biopsies did not have prognostic significance in predicting outcome in patients with FL who transformed. It is worth noting however, that the biopsies from the majority of patients with a survival above the median contained <15 macrophages per hpf. The location of the immune infiltrating cells in the diagnostic FL biopsies did not have prognostic significance in predicting survival outcome either. This is similar to the situation in de novo DLBCL where the immune microenvironment at diagnosis did not impact on survival outcome.

CHAPTER NINE:

Follicular Dendritic Cells and their role in Follicular Lymphoma

9.1 Introduction

The morphology of follicular lymphoma resembles a reactive germinal centre and the neoplastic cells are closely associated with non-neoplastic cells which are observed in normal reactive follicles. This includes both T lymphocytes and follicular dendritic cells (FDCs). The presence of FDC in non-malignant lymph nodes was first observed as a result of studies on the distribution of antigens within lymph nodes of rats (Nossal GJV et al. 1965). Injection of iodide labelled flagellin antigens showed distribution of the antigen in a diffuse pattern throughout the follicle within primary follicles. In secondary follicles, the antigen formed a cresentic shape in the superficial aspect of the follicle (Nossal GJV et al. 1965).

9.1.1 Functions of FDC

9.1.1.1 Architectural

FDC have many functions within non-malignant lymphoid tissue and provide a cellular architecture composed of long cytoplasmic processes which maintain the structure of the lymphoid follicle (Chen LL, Adams JC, Steinman RM 1978). Analysis of FDC within germinal centres in mouse spleen demonstrated that FDC have large nuclei often with two nuclear lobes with lack of heterochromatin. Visualisation by electron microscopy demonstrated a relative lack of cell body with the cytoplasm forming long arms which extended in several directions. These processes were flat in FDC positioned at the periphery of the nodule or deep within the germinal centre however at the junction of the mantle and germinal centre the processes became folded and coiled. The numbers and complexity of FDCs reduced on moving away from the mantle zone towards the middle of the germinal centre. There is also a lack of organelles particularly secretory granules (Chen LL, Adams JC, Steinman RM 1978).

9.1.1.2 Formation of Germinal Centres

FDCs are unique cells which present antigen to germinal centre B lymphocytes in the form of immune complexes composed of antigen and antibody (Kosco MH, Szakal AK, Tew JG 1988). FDCs express both Fc receptors (White RG et al. 1975; Heinen E et al. 1985) and complement receptor antigens, CR1, CR2 and CR3 (Reynes M et al. 1985). Immune complexes are captured by their complement receptors (Klaus GG, Humphrey JH 1977) and their FcγRs (Yoshida K, Van Den Berg TK, Dijkstra

CD 1993; Qin D et al. 2000) and presented to GC B lymphocytes which present antigen to T lymphocytes to obtain T lymphocyte help (Kosco MH, Szakal AK, Tew JG 1988). Some ICs are converted by FDCs into coated bodies, 'iccosomes' which can be endocytosed by GC B cells (Szakal AK, Kosco MH, Tew JG 1983). IC alone are poorly immunogenic (Muta T et al. 1994) suggesting that a function of FDCs is to convert poorly immunogenic IC into highly antigenic complexes to elicit a B cell response (Wu J et al. 1996). Expression of the IgG receptor FcγRIIB on murine FDC is essential for immune complex tapping, retention and conversion to a highly immunogenic form which elicits immune response (Qin D et al. 2000). Interaction of IC alone with B lymphocytes results in ITIM signalling and lack of B cell responsiveness. This is due to coligation of the B cell antigen receptor and B cell FcγRII (Diegel ML et al. 1994). The result of the coligation is reduction of cell proliferation (Pani G et al. 1995), inhibition of blastogenesis (Phillips NE, Parker DC 1984) and inhibition of immunoglobulin production (Ono M et al. 1996). The ability of IC on FDC to stimulate GC proliferation indicates that the interactions between IC and FDC can result in B cell activation. This may be as a result of rapid up-regulation of FDC FcγRII which minimizes IC-induced ITIM signalling (Aydar Y et al. 2004). Numerous adhesion molecules are present on FDCs which are essential to maintenance of FDC-B lymphocyte clusters including high levels of the adhesion receptors intercellular adhesion molecule 1 (ICAM-1 [CD54]) and vascular cell adhesion molecule 1 (VCAM-1), while the B lymphocytes express lymphocyte function-associated antigen 1 (LFA-1 [CD11a/18]), very late antigen 4 (VLA-4 [CD49d]), and CD44. Both the LFA-1/ICAM-1 and VLA-4/VCAM-1 adhesion pathways are involved in FDC-B lymphocyte binding (Koopman G et al. 1991). Soluble mediators are also important in germinal centre formation; interleukin-6 (IL-6) is produced by FDCs in the germinal centre and is important for formation of normal germinal centres and production of immunoglobulin G antibodies. This is dependent upon a C3 complement component (Kopf M et al. 1998).

9.1.1.3 Survival of Germinal centre B cells

FDCs are able to rescue germinal centre B lymphocytes from apoptotic cell death by physical contact (Lindhout E et al. 1993). This has been shown to be mediated through the LFA-1(CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways (Koopman G et al. 1994). In addition, stromally

produced sonic hedgehog protein is produced by FDCs and is essential for survival of germinal centre B cells (Sacedon R et al. 2005) and malignant lymphomas (Dierks C et al. 2007). Inhibition of the hedgehog pathway in lymphomas induced apoptosis via BCL-2 but was independent of p53 (Dierks C et al. 2007).

9.1.1.4 Activation, proliferation and differentiation of B lymphocytes

Culture of activated B lymphocytes with FDC demonstrated that the presence of FDC augmented B cell proliferation in a dose dependent manner. Depletion of the FDC abrogated this proliferation. This data supports the idea that FDC support B cell proliferation via costimulatory interactions (Burton GF et al. 1993). CD21 is essential for proliferation within GC. In murine models, high-affinity B lymphocytes which lack CD21 can be activated by antigen and enter GC but fail to proliferate. The expression of CD21 provides a survival signal for B lymphocytes within the germinal centre as well as reducing the threshold for B cell activation (Fischer MB et al. 1998). A monoclonal antibody against a human FDC molecule, 8D6, inhibits FDC-mediated costimulation of B cells as well as being able to inhibit growth of a lymphoma of GC origin which is dependent upon FDCs for growth. These findings indicate that a primary function of FDCs in GC is to stimulate B cell growth (Li L et al. 2000). Cytokines as well as direct cell contact are also important in stimulating proliferation; functionally active IL-15 is produced by human FDC in vivo and by an FDC cell line and is important for GC B cell proliferation (Park C-S et al. 2004). Human FDCs are also able to enhance cytokine-dependent growth and differentiation of CD40-activated B lymphocytes with high levels of immunoglobulin produced in cultures in the presence of FDC with IL-2 and IL-10 (Grouard G et al. 1995). In addition, FDC can help resting B lymphocytes to become effective antigen presenting cells by increasing levels of MHC class II molecules and CD86 (Kosco-Vilbois MH et al. 1993). Stromally produced sonic hedgehog protein is produced by FDCs and is essential for the survival, proliferation and antibody production by germinal centre B cells (Sacedon R et al. 2005).

9.1.2 Origin of FDC

The origin of FDCs remains unclear. Polymerase chain reaction analysis of single cells demonstrated that FDCs contain a very restricted mRNA pattern with high levels of message for the C3d receptor (CR2, Epstein Barr-virus/EBV receptor,

CD21) and lack of mRNA for CD20, CD45, CD4, fibronectin, and platelet-derived growth factor receptor alpha and beta. These observations are consistent with the hypothesis that FDCs may not be of classical haematopoietic or fibroblastic origin (Schreiver F, Freeman G, Nadler LM 1991). Another hypothesis is that antigen transporting cells, which can trap immune complexes on their cell surface and move them into lymph nodes (Szakal AK, Holmes KL, Tew JG 1983) and are reactive with monoclonal antibody produced against FDC (Kosco MH, Pflugfelder E, Gray D 1992) may be FDC precursors. However, the monoclonal antibody KiM4 which is specific for human FDC reacts with a mononuclear cell within blood suggesting that these cells are FDC precursors. As the KiM4 antibody was raised against a macrophage cell line this suggested that FDCs may be of haematopoietic cell origin (Parwaresch MR et al. 1983). Work in severe combined immunodeficiency (SCID) mice showed that FDC of donor phenotype can be found in SCID mice after reconstitution with primary lymphoid tissue from mice or rats. This suggests that FDC can be derived from precursors in primary lymphoid tissues which migrate into secondary lymphoid tissue and that maybe FDC can be derived from local stromal cells (Kapasi ZF et al. 1998). The successful isolation and maintenance of FDC cell lines isolated from human tonsils in fibroblast medium together with the expression of alpha smooth muscle action and STRO-1 suggested that FDC are closely related to bone marrow stromal cell progenitors. The expression of alpha smooth muscle actin indicates that FDC are related to myofibroblasts and suggests that they may represent a specialised form (Muñoz-Fernández R et al. 2006). The development of FDCs and their niche is dependent upon activated B lymphocytes in the absence of T lymphocytes and is driven by lymphotoxin-alpha (Gonzalez M et al. 1998; Fu Y-X et al. 1998). Once FDC clusters, FDC surrounded by B cells, are formed by the lymphotoxin alpha expressing B cells, T cells can interact with the B cells to generate GCs and allow class switching to occur (Fu Y-X et al. 1998). Recent data from murine studies has demonstrated activation of FDCs by T lymphocytes via CD40 and T cell lymphokines (Selvakumar S et al. 2006). This would be in keeping in humans with hyper-IgM syndrome whose T lymphocytes lack CD40 ligand and in whom GC do not form (Facchetti F et al. 1995).

9.1.3 Immunophenotype of FDC

As mentioned previously, FDCs contained high levels of mRNA for CD21, the

complement receptor C3d (CR2, Epstein Barr-virus/EBV receptor) (Schreiver F, Freeman G, Nadler LM 1991). Immunostaining using CD21 reveals a web-like staining pattern over the entire area of the GC and sometimes extends in the marginal zone (Maeda K et al. 2002). Human follicular dendritic cells also express CR1 and CR3 complement receptor antigens as well as Cr2 (Reynes M et al. 1985). There are several other monoclonal antibodies which have been identified as showing relatively specific staining for FDCs. CNA.42 monoclonal antibody recognises a 120-kd glycosylated antigen which is formalin resistant and which is mainly expressed by FDCs (Raymond I et al. 1997). The staining pattern in reactive lymph nodes of both Ki-M4p and CNA.42 was a web-like pattern within the germinal centre and/or mantle zones (Maeda K et al. 2002). The monoclonal antibodies against human low-affinity Fcε receptor (FcεRII/CD23) display a web-like immunoreactivity with GC of reactive lymphoid follicles but with expression restricted to the light zone especially the apical area with no staining observed in the dark zone (Maeda K et al. 2002). In routine diagnostic immunocytochemistry CD21 and CD23 are used as immunophenotypic markers of FDCs. CD35 is also a complement receptor and binds C3b and C4b. CD35 has an inhibitory effect on complement activation (Fearon DT, Ahearn JM 1989) and generates ligands for CD21 by catalyzing the conversion of C3b. The staining pattern for CD35 in reactive lymph nodes demonstrates a web-like pattern extended throughout the area of the GC (Maeda K et al. 2002).

Analysis of human fetal and adult lymph nodes has demonstrated an immunophenotypic alteration of FDC during development. The antigens CNA.42 and Ki-M4p were expressed in a small number of cells with dendritic morphology in underdeveloped lymph nodes from the foetus of gestational ages 19-22 weeks. In foetal lymph nodes aged 25-40 gestational weeks primary lymphoid follicles were present and FDC within these expressed CD21, CD35 and NGFR as well as CNA.42 and Ki-M4p. In addition, variable expression of CD23 was seen. The presence of CD23 implies that these cells are able to capture and retain immune complexes, an essential function of FDC. These molecules were termed ‘essential’ FDC molecules. Additional molecules were also expressed in the FDC of full developed secondary lymphoid follicles; CD55, VCAM-1 and S100α. These were referred to as activated FDC molecules implying an activated state (Kasajima-Akatsuka N, Maeda K 2006).

Immunostaining for S100α demonstrated an intense cytoplasmic and nuclear stain in FDCs mainly in the dark zone of the GC (Maeda K et al. 2002).

9.1.4 FDC and lymphoma

The intimate association and requirement for FDC in GC reactions would suggest that these cells are also important in follicular lymphoma; a germinal centre derived lymphoma which morphologically resembles a reactive germinal centre, as well as other haematological malignancies. The presence of FDC in lymphomas outside the lymphoid system implies that their presence is essential to survival of the neoplasm. Studies in murine models have demonstrated that the induction of apoptosis as a result of anti-Fas, etoposide, cyclophopshamide and busulfan was significantly reduced by the presence of FDC at ratios of FDC to B lymphocytes as low as 1:16. Prevention of apoptosis was not due to alterations in Bcl-2, Bax or Bcl-X_L. Conversely the ability of adriamycin to cause apoptosis was not affected by the presence of FDC. This data suggests that FDC can protect B lymphoma cells from Fas induced cell death. (Schwarz YX et al. 1999). This may be important in mechanisms of drug resistance; there is evidence which indicates defective activation of the CD95 (APO-1/Fas) pathway may cause drug resistance in human leukaemic cells. Indeed some chemotherapeutic agents have been shown to up-regulate Fas levels in leukaemia cells (Friesen C et al. 1996). In humans, proliferation of NHL lymphocytes in vitro has been demonstrated to be dependent upon FDC interactions (Petrasch S et al. 1992). In Hodgkins lymphoma FDCs have been shown to be associated with prognosis; retrospective analysis of 102 patients with the most common types of Hodgkins disease (nodular lymphocyte predominant, nodular sclerosing and mixed cellularity) demonstrated that FDC status in the neoplastic areas was prognostically important with a positive FDC status predicting a favourable response and a negative FDC status an unfavourable one (Alavaikko MJ et al. 1994). It is known that FDC secrete CXCL13 (Ansel KM et al. 2000) which binds to the receptor CXCR5, expressed on mature B cells and a subpopulation of T-helper memory cells (Dobner T et al. 1992), FL cells have been shown to express CXCR5 and migrate in response to CXCL13. In addition a synergistic effect between CXCL13 and CXCL12, a chemokine produced by stromal cells in lymphoid tissue, was observed. This is postulated to be important in the accumulation of FL cells within specific anatomic sites (Husson H et al. 2002). In addition, FDCs have been

demonstrated to produce monocyte chemoattractant protein-1 (MCP-1) which may induce chemotaxis of FL cells (Husson H et al. 2001).

9.1.5 Clusterin

Clusterin is a glycoprotein encoded by a gene in the region 8p21 (Purrello M et al. 1991). The protein is present in many tissues and all human fluids and has roles in many diverse human processes. There are two different isoforms of clusterin, a secreted form sCLU, which is a glycosylated protein of 76-80kDa and appears as two bands on Western blot. A band at 60kDa represents the full length protein and a second 40kDa smear is also seen (Leskov KS et al. 2003). The second form is a nuclear isoform which is produced as a 49kDa protein (pnCLU) and is located in the cytoplasm of human cells (Jin G, Howe PH (1999). As a result of cell damage, the pnCLU protein is modified and a mature 55kDa proapoptotic form is generated which can induce apoptosis (Leskov KS et al. 2003). It has been proposed that tumour cell survival is connected with overexpression of sCLU and loss of nCLU (Pucci S et al. 2004). Clusterin inhibits apoptosis by interacting with activated Bax preventing release of cytochrome c from mitochondria and caspase activation (Zhang H et al. 2005). Overexpression of sCLU has been shown in many epithelial tumours including prostate cancer (Scaltriti M et al. 2004), lung cancer (July LV et al. 2004), colon carcinoma (Pucci S et al. 2004), and breast carcinoma (Redondo M et al. 2000). sCLU has been demonstrated to be a sensitive marker for both human and murine intestinal tumours suggesting that its expression may be a potential biomarker (Chen X et al. 2003). In addition, clusterin has been demonstrated to be a marker for anaplastic large cell lymphoma although an association with prognosis has not been evaluated. The same study demonstrated the expression of clusterin in residual FDC in Hodgkins lymphoma (Nascimento A et al. 2004). Clusterin expression has also been observed in some DLBCL neoplastic cells (Saffer H et al. 2002). Clusterin has been demonstrated to be an FDC marker and possibly an FDC-derived trophic factor for GC B cells (Huber C et al. 2005). Drugs which silence clusterin using antisense oligonucleotides and short interfering double-stranded RNAs (si RNAs) chemosensitise human lung adenocarcinoma cells both *in vitro* and *in vivo* and indicate that clusterin is a valid therapeutic target for cancer therapy (July LV et al. 2004). A single study has examined clusterin expression in FL as part of a larger study of malignant lymphomas. Twenty four cases of FL were examined and staining

of tumour cells was not observed. The presence of staining in FDC was observed (Saffer H et al. 2002).

9.1.6 Aims

The aim of this work was to study in closer detail the FDC present in FL samples and their impact on the natural history of FL. This involved analysis of immunophenotype of FL biopsies and comparison of the immunophenotype to that of reactive lymph nodes using double immunofluorescence. Subsequently, immunophenotypic markers, including clusterin, were assessed by immunocytochemistry on TMA of FL to determine if their absence or presence had any effect on outcome (overall survival and time to transformation) in patients and as such could be used as a biomarker. In addition, gene expression profiling of paired pre- and post-transformation samples identified down-regulation of CD21 and CD23 on transformation. Protein confirmation of the gene expression changes on transformation was obtained by immunocytochemistry of paired pre- and post-transformation biopsies.

9.2 Materials and Methods

9.2.1 Patients

Three groups of patients were identified to investigate the role of FDCs in the natural history of FL. A cohort of 94 patients with diagnostic paraffin embedded FL biopsies in the archives of St Bartholomew's Hospital was identified. The patients were selected on the basis of availability of paraffin embedded tissue and clinical data. From this primary cohort of 94 patients a second cohort of twenty six samples were identified from patients who transformed from FL to DLBCL in less than 3 years from initial diagnosis and twenty five samples were identified from patients who transformed after eight years from initial diagnosis. A third cohort of patients with FL who subsequently transformed to DLBCL was identified. In this cohort of patients paired pre and post transformation samples (n=126) were obtained from 34 patients. Ethical approval for this study was obtained from the local regional ethics board.

9.2.2 Immunofluorescence

Full sections from five reactive lymph nodes and five diagnostic follicular lymphoma

lymph nodes were cut to a thickness of 4 microns and applied to TESPA-coated slides, then dewaxed and blocked in hydrogen peroxide/methanol solution. Antigen retrieval was performed by pressure-cooking in citrate (Norton AJ, Jordan S, Yeomans P 1994). Sections were then stained using the Vector M.O.M immunodetection kit (Vector Laboratories, Inc, California). Appropriate negative controls were always used. The antibodies used and the dilutions are listed in **Table 31**.

Table 31 Antibody sources, dilutions and antigen retrieval

Antibody	**Clone**	**Source**	**Dilution**	**Method of Antigen Retrieval**
CD21	NCL-CD21-2G9	Novocastra, Newcastle, UK	1/50 for 40 minutes	Pressure cooking citrate
CD23	NCL-CD23-IB12	Novocastra, Newcastle, UK	1/50 for 40 minutes	Pressure cooking citrate

9.2.2.1 Analysis of immunofluorescence stained sections

Twenty secondary follicles from reactive lymph nodes were selected at random and were assessed at original magnification of x10. Twenty neoplastic follicles were selected at random from the FL lymph nodes ensuring all areas of the node were represented and analysed at an original magnification X10.

9.2.3 Gene expression profiling

Lymphochip cDNA microarrays were used to quantitate mRNA expression according to previously described methodology (Alizadeh et al. 2000; Rosenwald et al. 2002). A list of genes that significantly differed between FL and DLBCL was constructed using the random variance model *t*-test (Wright and Simon 2003), with the cut off value for *P* set at <0.001. The probability of belonging to a particular sub-class of DLBCL was determined using the Bayesian statistical predictor and clone set previously reported (Wright et al. 2003).

9.2.4 Construction of TMAs and Immunocytochemistry

Tissue microarrays (TMA) were constructed of 1mm cores of patient tissue taken from representative areas of lymphoma (>5 neoplastic follicles) using pre-marked H&E stained sections and from reactive tonsil and appendix controls, in triplicate using a manual arrayer (Beecher Scientific, Silver Spring, MD) as previously described (Hedvat CV et al. 2002; Kononen J et al. 1998). Approximately 120 cores were applied per slide.

The test panel of antibodies and dilutions used for immunocytological analysis are shown in **Table 32**. TMA blocks were cut to 4 microns and applied to TESPA-coated slides, then dewaxed and blocked in hydrogen peroxide/methanol solution. Antigen retrieval was performed by pressure-cooking in citrate (Norton AJ, Jordan S, Yeomans P 1994). Sections were then stained using the Vector Elite ABC kit (PK6100, Vector Laboratories, Peterborough, UK), followed by diaminobenzidine chromogen (Biostat, Stockport, UK). Appropriate negative and positive controls were always used.

Table 32 Table of antibodies used, dilution and method of antigen retrieval

Antibody	**Clone**	**Source**	**Dilution**	**Method of antigen retrieval**
CD21	NCL-CD21-2G9	Novocastra, Newcastle, UK	1/50 for 40 minutes	Pressure cooking citrate
CD23	NCL-CD23-IB12	Novocastra, Newcastle, UK	1/50 for 40 minutes	Pressure cooking citrate
CD35	Ber-MAC-DRC	Dako UK Ltd, Cambs, UK	1/50 for 40 minutes	Pronase
CAN.42	CNA.42	Dako UK Ltd, Cambs, UK	1/50 for 40 minutes	Pressure cooking
Clusterin	7D1	Novocastra, Newcastle, UK		Pressure cooking

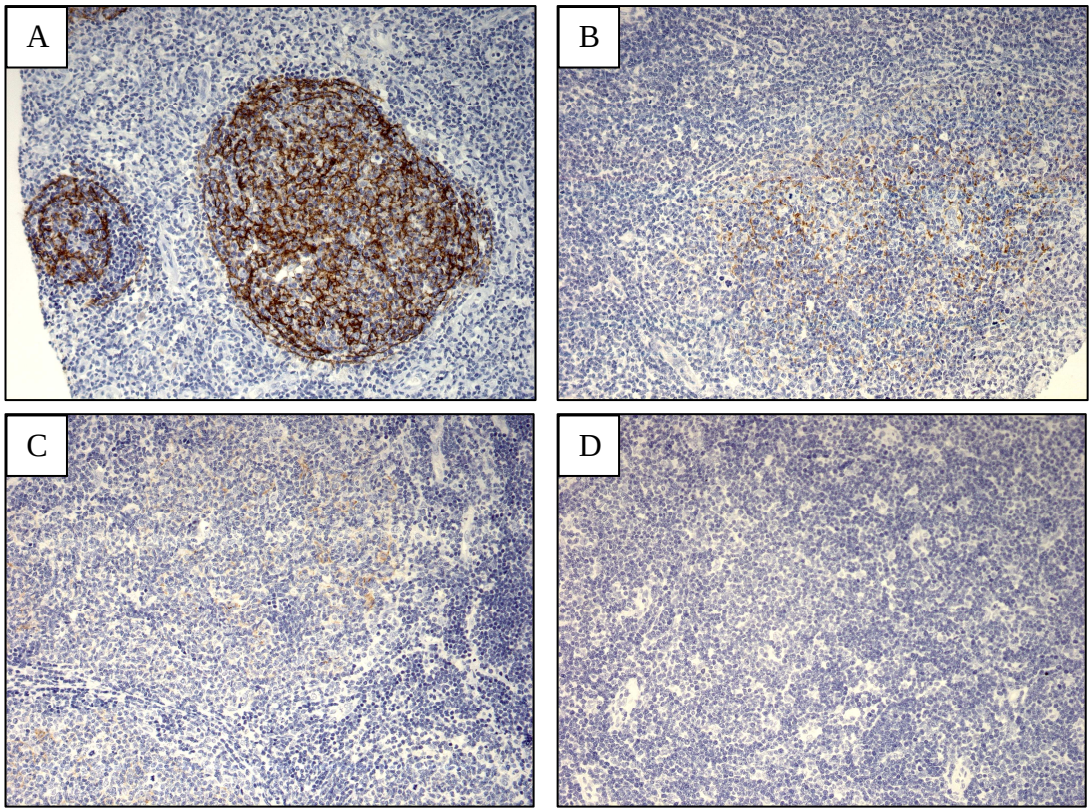
9.2.5 Immunocytochemical analysis

Presence of adequate FL tumour cells in the tissue cores was confirmed by scoring with a diagnostic lymphoma antibody panel comprising CD3, CD5, CD10, CD20, CD21, CD23, BCL-2, BCL-6, Ki-67, TP53, MUM-1 as previously described in Chapter 4.

The entire 1mm diameter core was analysed at original magnification x5 initially and then at high power (x40 magnification) in each case. Where one of three cores could not be scored due to insufficiency of tissue, consensus of 2 cores was accepted although this was necessary in only 10% of the total cores. If two cores of three were insufficient, no scoring was attempted and the result was noted as ‘absent’ for that sample with the antibody in question.

The FDC meshwork was scored into four categories based on pattern as shown in **Figure 47.**

Figure 47 Immunocytochemistry for CD21 demonstrating four patterns of staining which were used for scoring. A) Well formed meshwork, B) Sparse meshwork, C) A few fragments of mesh remaining, D) negative. All photomicrographs are at original magnification x40



Meshwork was defined as a web-like immunoreactivity corresponding to the lymphoid follicles (Maeda K et al. 2002).

9.2.6 Western Blotting of tonsil sample and FL samples using anti-human polyclonal antibody to Clusterin

A tonsil sample and two FL samples were identified and the protein was extracted using 200 μL of extraction /labelling buffer (Clontech Lab Inc.). The amount of protein analysed using the BCA™ Protein Assay Kit (Pierce, Rockford, IL). Fifteen micrograms of protein was suspended with loading buffer and reducing agent and denatured by heating at 95˚C for ten minutes. The mixture was loaded onto the gel and run for 35 minutes at 200V. The gel was transferred to a nitrocellulose membrane and blocked for one hour with blocking solution. The primary antibody was incubated overnight at 4˚C at a dilution of 1:200. The secondary antibody was applied at a dilution of 1:2000 for two hours. A housekeeping antibody, GAPDH, was analysed to ensure equal protein loading of wells.

9.3 Results

9.3.1 The distribution of FDC meshwork is different in reactive lymph nodes as compared to FL

Double immunofluorescence for CD21 and CD23 was performed on full sections of reactive lymph nodes (n=5) and FL lymph node biopsies (n=5). In the secondary follicles of all the reactive lymph node sections CD21 was observed in a diffuse mesh located throughout the germinal centre in the areas of the dark zone, basal light zone and apical light zone. CD23 was observed in a polarized band located around the follicle edge in the apical light zone (**Figure 48**). These findings are in keeping with those observed by Hardie et al. (1993) who used immunofluorescence to analyse molecules distinguishing functional compartments in germinal centres (Hardie DL et al. 1993).

In all the FL sections analysed there was loss of polarity of CD23 with a CD23 positive meshwork present throughout the neoplastic follicle. A CD21 positive meshwork was also present throughout the follicle as seen in reactive lymph nodes (**Figure 49**).

Figure 48 Immunofluorescence images stained with CD21 monoclonal antibody (green) and CD23 monoclonal antibody (red) of secondary follicles in a reactive lymph node demonstrating, A) Presence of CD21 meshwork throughout the GC, B) CD23 meshwork polarized to the apical light zone of the GC, C) Merged images of CD21 and CD23 demonstrating some areas of coexpression of markers depicted as orange colouration. All images OM x10

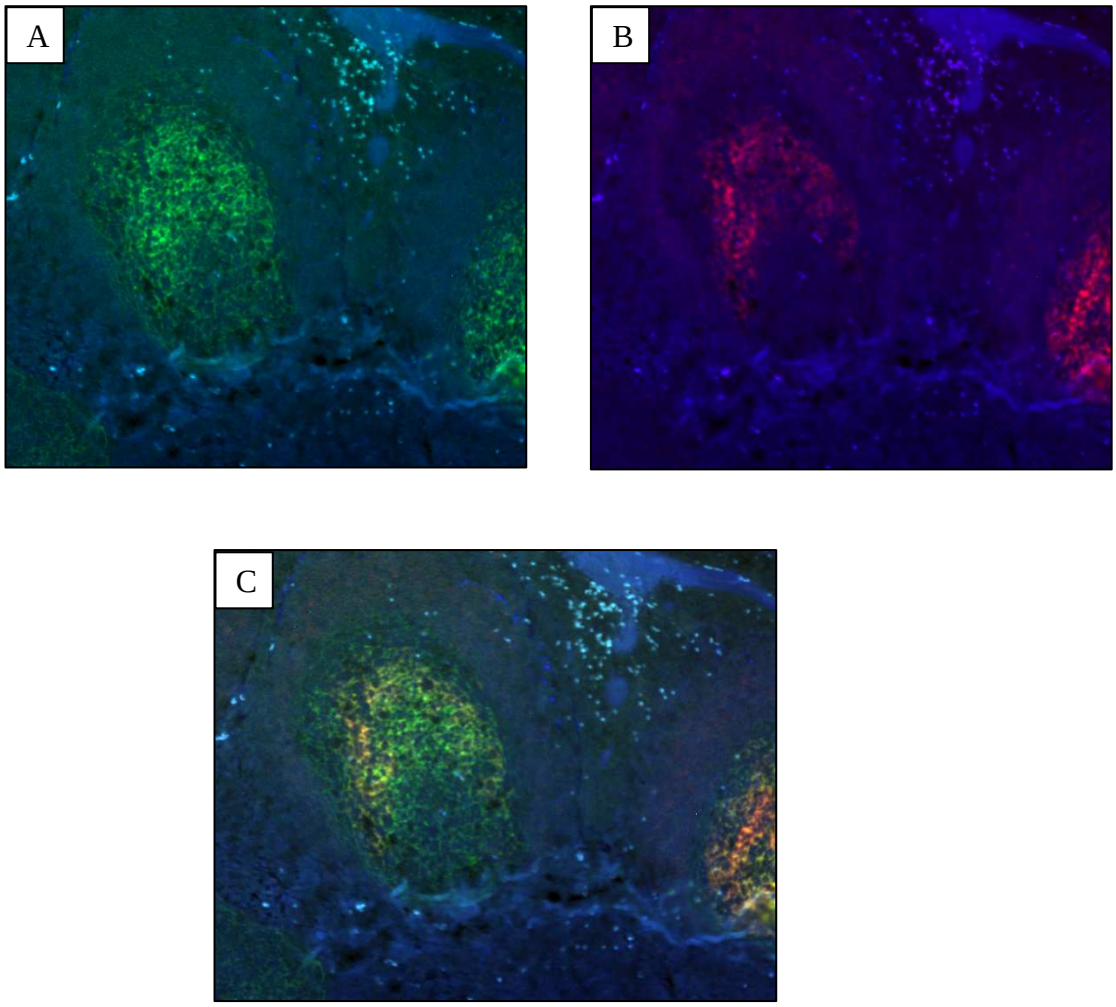
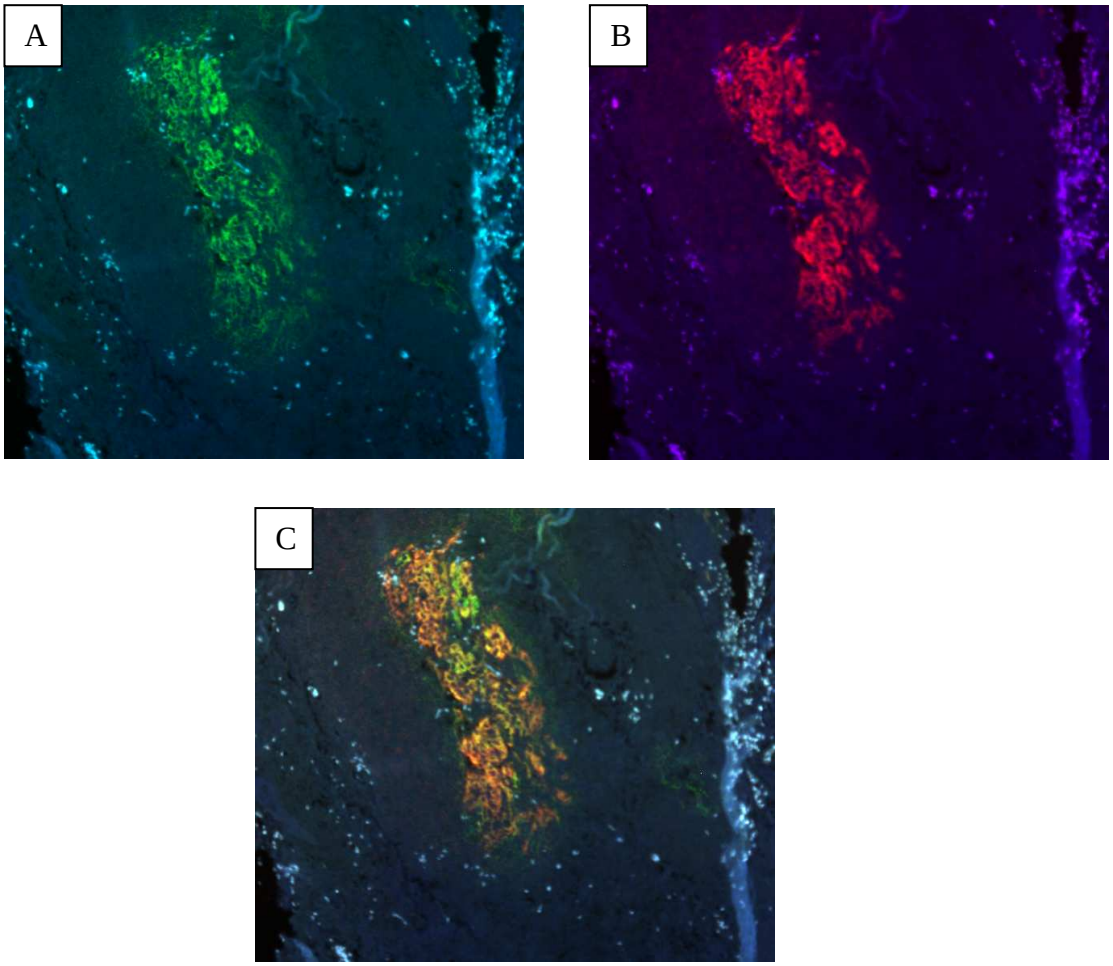


Figure 49 Immunofluorescence images stained with CD21 monoclonal antibody (green) and CD23 monoclonal antibody (red) of neoplastic follicles in lymph node of FL demonstrating, A) Presence of CD21 meshwork throughout the neoplastic follicle, B) CD23 meshwork throughout the neoplastic follicle, C) Merged images of CD21 and CD23 demonstrating areas of coexpression of markers depicted as orange colouration. All images OM x10



9.3.2 Analysis of immunophenotype of FDC in TMAs composed of diagnostic lymph node biopsies from patients with FL

A cohort of ninety four patients diagnosed with FL on whom initial diagnostic biopsies were available was identified. Histology was reviewed in all cases. The patient characteristics are detailed in **Table 33**

Table 33 Patient Characteristics of 94 patients diagnosed with FL

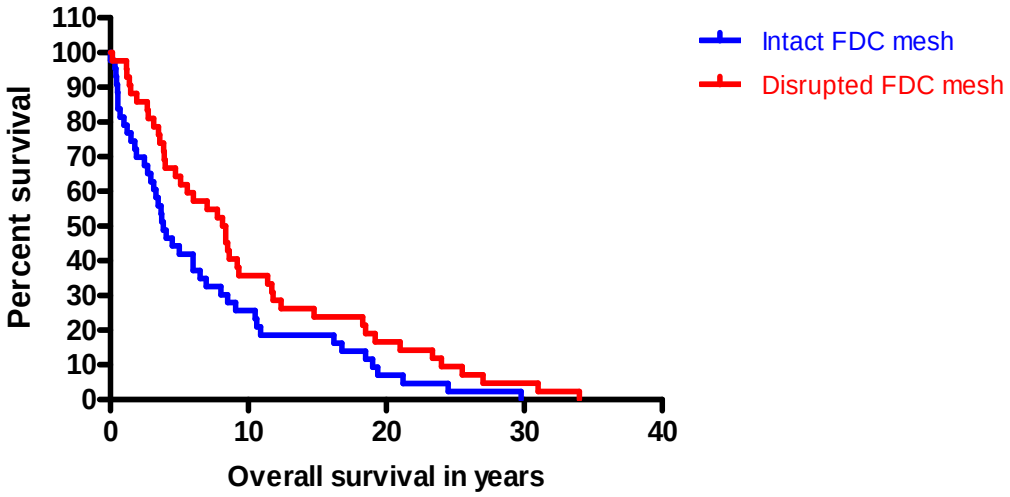
	Patients with FL (n=94)
Sex	Male = 60 Female =34
Median age (years)	56 (range 27 – 80)
Grade at diagnosis	Grade 1 =39 Grade 2 = 33 Grade 3a = 15
Stage at diagnosis	Stage 1 = 6 Stage 2 = 11 Stage 3 = 19 Stage 4 = 53
Median OS (years)	5.10 (range 0.02 – 34.86)
Proceeded to transformation	31/94 (33%)

9.3.2.1 Analysis of FDC meshwork using immunocytochemistry for CD21

Eighty five cases from a possible ninety four were evaluable for the analysis of CD21 staining of FDC due to core loss from the TMA. Scoring was originally performed as indicated in the materials and methods however the three categories; sparse meshwork, fragmented meshwork and negative were combined into a single category termed disrupted meshwork. The well formed meshwork category remained as described in the materials and methods. This brought the scoring system in line with that used by Glas et al. (Glas AM et al. 2007) in their analysis of FDC. Forty two cases (49%) demonstrated a disrupted meshwork and forty three (51%) demonstrated a well formed meshwork. The overall survival of patients with a non disrupted FDC meshwork as assessed by CD21 expression was worse (median 3.7

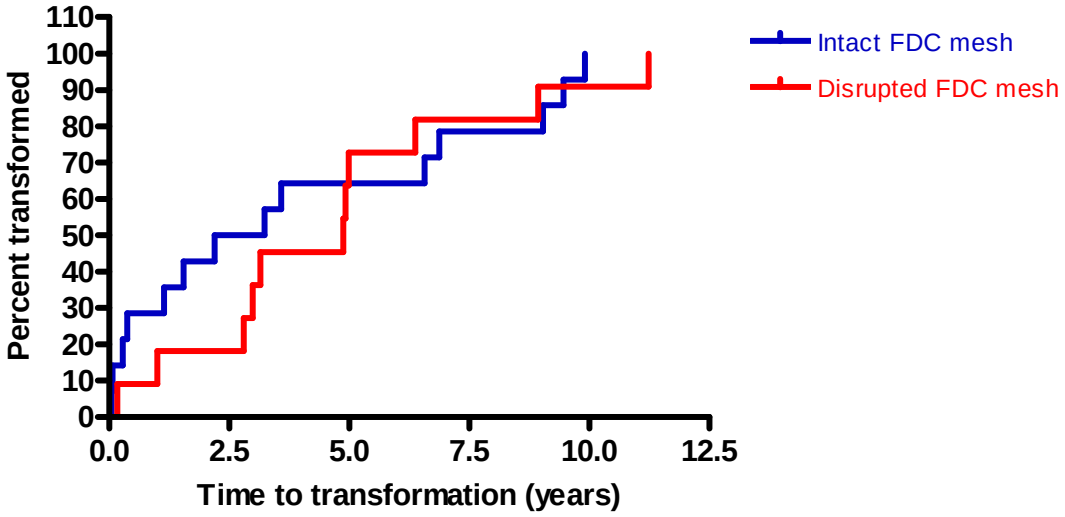
years from diagnosis) than patients with a disrupted FDC meshwork at diagnosis (median 8.15 years from diagnosis; p=0.15) although this was not significant (**Figure 50**).

Figure 50 Overall survival in years, of patients with FL, based on integrity of FDC meshwork assessed by CD21 expression in initial diagnostic biopsies. The blue line indicates patients with a preserved FDC meshwork in the initial diagnostic FL biopsy (n=43). The red line indicates patients with a disrupted FDC meshwork in the initial diagnostic FL biopsy (n=42).



Transformation to DLBCL occurred in twenty six assessable cases. The median time to transformation was 3.07 years from diagnosis. The presence of an intact or disrupted FDC meshwork as assessed by CD21 immunocytochemistry was not predictive of likelihood of transformation (p=0.7) (**Figure 51**).

Figure 51 Time to transformation in years, of patients with FL, based on integrity of FDC meshwork assessed by CD21 expression in initial diagnostic biopsies.The blue line indicates patients with a preserved FDC meshwork in the initial diagnostic FL biopsy (n=15). The red line indicates patients with a disrupted FDC meshwork in the initial diagnostic FL biopsy (n=11).



9.3.2.2 Analysis of FDC meshwork using immunocytochemistry for CD23

Eighty cases from a possible ninety four were evaluable for the analysis of CD23 staining of FDC. Scoring was performed as described for CD21. Fifty six cases (70%) demonstrated an intact FDC meshwork and twenty four cases (30%) disrupted FDC meshwork. There was no difference between the median overall survival of patients with an intact FDC meshwork and patients with a disrupted FDC meshwork, as assessed by CD23 staining in the initial diagnostic FL biopsy (p=0.13).

Transformation to DLBCL occurred in twenty five assessable cases. The median time to transformation from diagnosis was 2.99 years. There was no difference in the median time to transformation for patients with an intact FDC meshwork (n=16) and patients with a disrupted FDC meshwork (n=9), as assessed by CD23 staining in the initial diagnostic FL biopsy (p=0.7).

9.3.2.3 Analysis of FDC meshwork using immunocytochemistry for CD35

Seventy three cases from a possible ninety four were evaluable for the analysis of CD35 staining of FDC. Scoring was performed as described for CD21 and CD23. Sixty two cases (85%) demonstrated a disrupted FDC meshwork and eleven cases (15%) an intact FDC meshwork. There was no difference between the median overall survival of patients with an intact FDC meshwork and patients with a disrupted FDC meshwork, as assessed by CD35 staining in the initial diagnostic FL biopsy (p=0.48)(**Figure 52**).

Transformation to DLBCL occurred in twenty five assessable cases. The median time to transformation was 3.2 years. Twenty three patients (92%) with a disrupted FDC meshwork transformed to DLBCL compared to 2 patients (8%) with an intact FDC meshwork (p=0.01). The median time to transformation of patients with an intact FDC meshwork as assessed by CD35 staining was 0.38 years compared to 3.59 years for patients with a disrupted FDC meshwork (p=0.09). It should be noted that only two cases demonstrated an intact FDC meshwork (**Figure 53**).

Figure 52 Overall survival in years, of patients with FL, based on integrity of FDC meshwork assessed by CD35 expression in initial diagnostic biopsies.The blue line indicates patients with a preserved FDC meshwork in the initial diagnostic FL biopsy (n=11). The red line indicates patients with a disrupted FDC meshwork in the initial diagnostic FL biopsy (n=62).

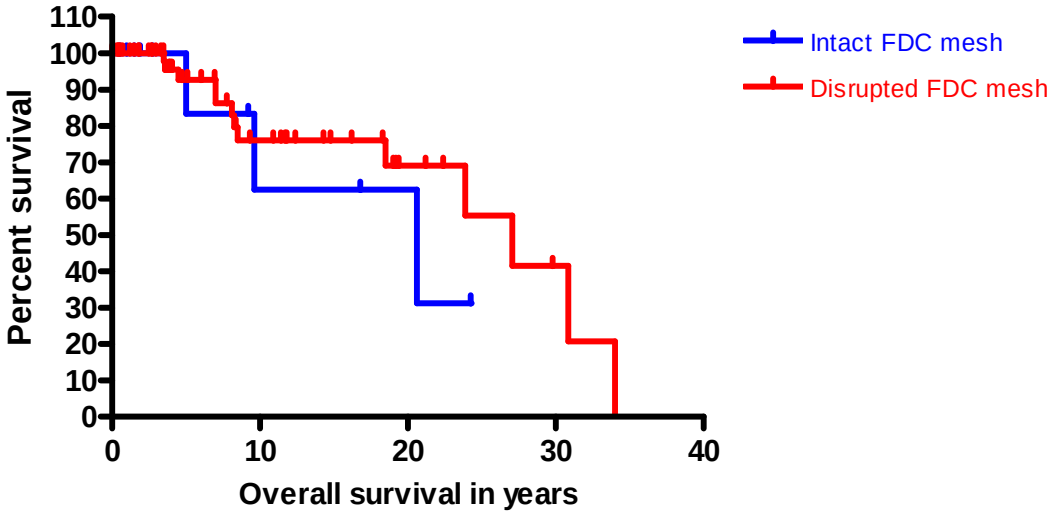
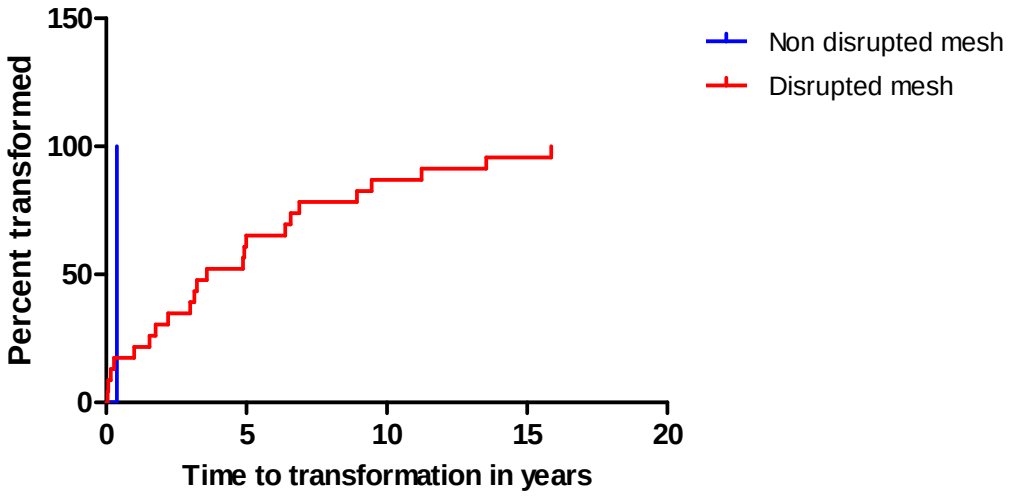


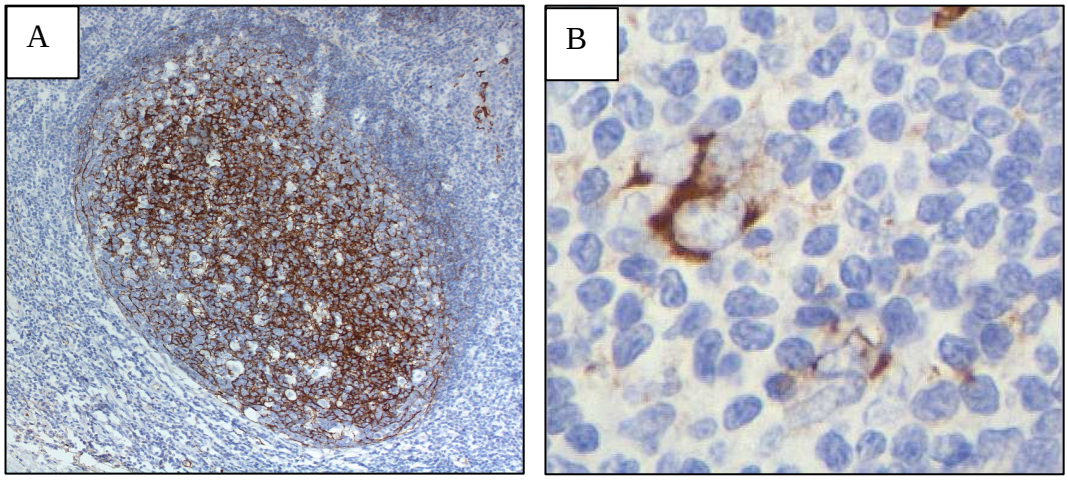
Figure 53 Time to transformation, in years, of patients with FL based on integrity of FDC meshwork assessed by CD35 expression in initial diagnostic biopsies.The blue line indicates patients with a preserved FDC meshwork in the initial diagnostic FL biopsy (n=2). The red line indicates patients with a disrupted FDC meshwork in the initial diagnostic FL biopsy (n=23).



9.3.2.4 Analysis of FDC meshwork using immunocytochemistry for Clusterin

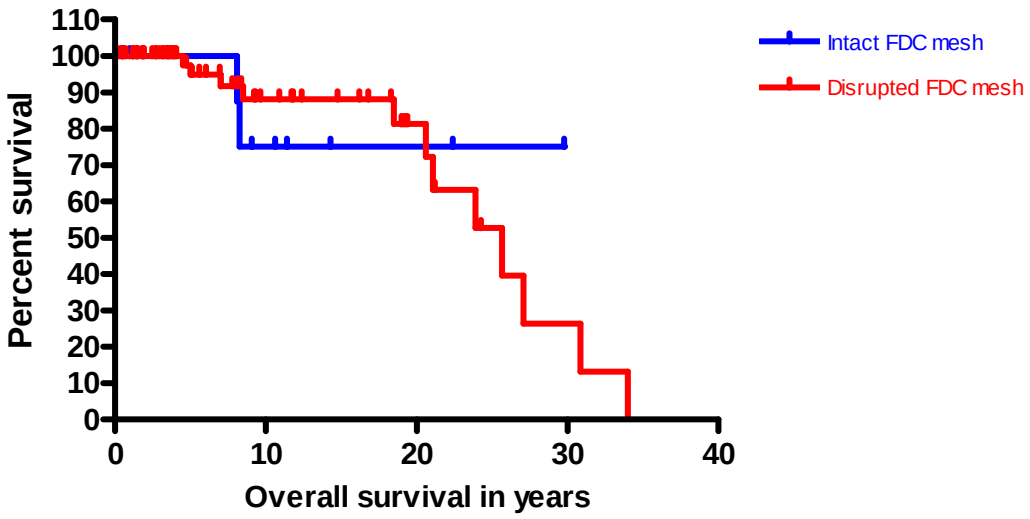
Clusterin is not a traditionally used immunocytochemical marker to highlight FDC however on application to numerous reactive lymph nodes and lymphoid tissue it was observed to highlight the FDC meshwork (**Figure 54**)

Figure 54 Immunocytochemistry for Clusterin performed on a reactive lymph node demonstrating, A) Low power image depicting positive FDC meshwork within a reactive lymphoid follicle, original magnification x10, B) High power image depicting positive staining of FDC within a reactive lymph node, original magnification x40



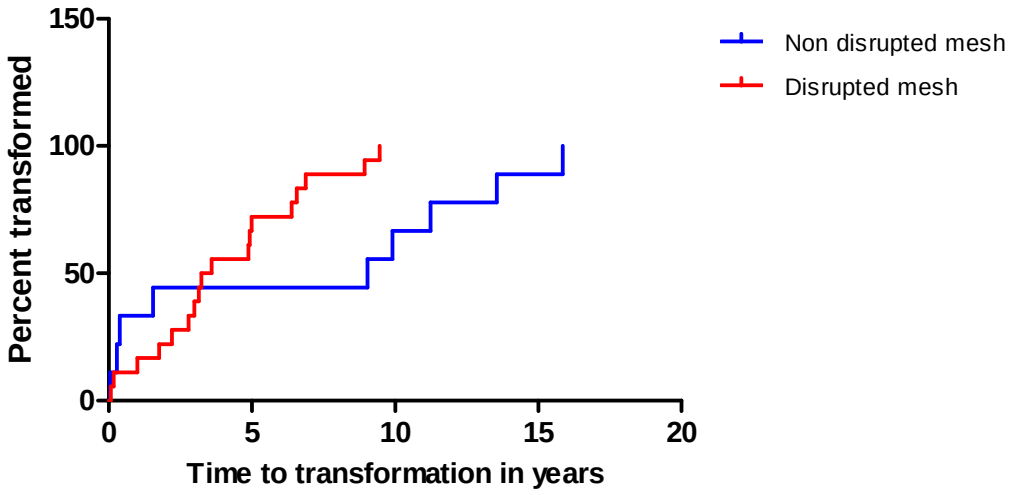
Consequently, clusterin staining of FDC in FL was analysed in the same way as previous described. Eighty three cases from a possible ninety four were evaluable, sixty nine cases (83%) demonstrated a disrupted FDC meshwork and fourteen cases (17%) demonstrated an intact FDC meshwork. The median overall survival of patients whose initial diagnostic biopsy demonstrated an intact FDC meshwork was 8.18 years. The median overall survival of patients whose initial diagnostic biopsy contained a disrupted FDC meshwork was 5.1 years. Overall survival was not statistically significant (p=0.8).

Figure 55 Overall survival in years, of patients with FL, based on integrity of FDC meshwork assessed by Clusterin expression in initial diagnostic biopsies. The blue line indicates patients with a preserved FDC meshwork in the initial diagnostic FL biopsy (n=14). The red line indicates patients with a disrupted FDC meshwork in the initial diagnostic FL biopsy (n=69).



Transformation to DLBCL occurred in twenty eight assessable cases. The median time to transformation of patients with an intact FDC meshwork as assessed by Clusterin staining was 9.04 years compared to 3.4 years for patients with a disrupted FDC meshwork (p=0.04) (**Figure 56**).

Figure 56 Time to transformation, in years, of patients with FL based on integrity of FDC meshwork assessed by Clusterin expression in initial diagnostic biopsies. The blue line indicates patients with a preserved FDC meshwork in the initial diagnostic FL biopsy (n=10). The red line indicates patients with a disrupted FDC meshwork in the initial diagnostic FL biopsy (n=18).



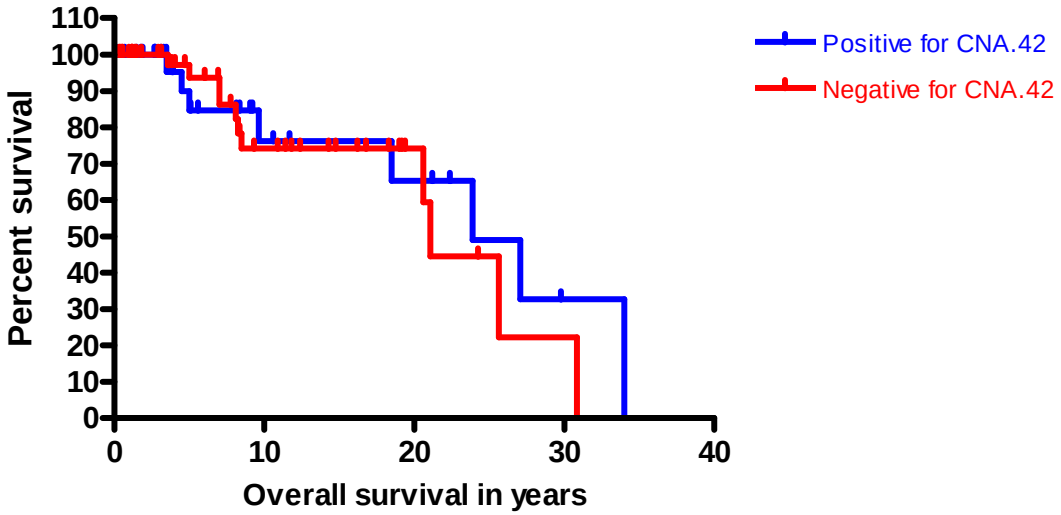
9.3.2.5 Analysis of FDC meshwork using immunocytochemistry for CNA.42

Eighty cases of a possible ninety four were evaluable for the expression of CNA.42. Initially scoring of CNA.42 staining was performed as described previously however, only two cases of intact meshwork were observed. This is strikingly different to reactive lymph nodes and reactive lymphoid tissue where the FDC meshwork within reactive germinal centres is clearly highlighted by CNA.42. Consequently CNA.42 was scored on the presence or absence of FDC stained positively.

Forty eight cases (60%) were negative for CNA.42 staining and thirty two cases

(40%) contained FDCs stained positively for CNA.42. The median overall survival of patients with biopsies which contained positively stained FDC was not significantly different to patients with biopsies which were negative for CNA.42 (5.05 years versus 7 years)(p=0.6) (**Figure 57**).

Figure 57 Overall survival in years, of patients with FL, based on staining of FDC by CNA.42 in initial diagnostic biopsies. The blue line indicates patients with a positively stained FDC present in the initial diagnostic FL biopsy (n=32). The red line indicates patients without positively stained FDC in the initial diagnostic FL biopsy (n=48).



9.3.2.6 The minority of FDC in FL are mature as determined by mmunocytochemical staining for CD21, CD23 and CD35

According to the developmental model of immunophenotypic differences in FDC developed by Kasajima-Akatsuka and Maeda; mature FDC are designated as expressing CD21, CD23 and CD35 (Kasajima-Akatsuka.N, Maeda K 2006). Only six cases of 94 (6.4%) in this study demonstrated an intact FDC meshwork stained by immunocytochemistry for CD21, CD23 and CD35 and would consequently be classified as mature. The finding that a minority of FL cases contain FDC of mature immunophenotype has previously been observed although the percentage of cases with a mature immunophenotype was much higher (40%) (Chang K et al. 2003) than in my study.

The results for patterns of immunocytochemical staining are detailed in **Table 34**.

Table 34 Table to demonstrate the immunocytochemical pattern of staining of meshwork using monoclonal antibodies to CD21, CD23, CD35 and Clusterin

Antibody	**Intact Meshwork**	**Disrupted Meshwork**			**Total**
		Sparse	**Fragments**	**None**	
CD21	43	17	12	14	86
CD23	56	11	8	5	80
CD35	11	10	18	34	73
Clusterin	14	15	40	14	83

9.3.2.7. The number of macrophages in FL is not related to the integrity of the FDC meshwork

FDC produce monocyte chemoattractant protein-1 (MCP-1) which may recruit FL cells (Husson H et al. 2001). The secretion of these factors may also be responsible for the varied immune cells infiltrates present in the FL microenvironment. The presence of an intact FDC meshwork may provide an increased stimulus for the recruitment of macrophages, a known adverse prognostic factor (Farinha P et al. 2005), to the neoplastic follicles via MCP-1. Using the macrophage counting criteria of Farinha et al. (Farihna P et al. 2005), the number of macrophages present was correlated with the integrity of the FDC meshwork. There was no association between the integrity of the FDC meshwork and the number of CD68 positive macrophages present in the FL biopsies.

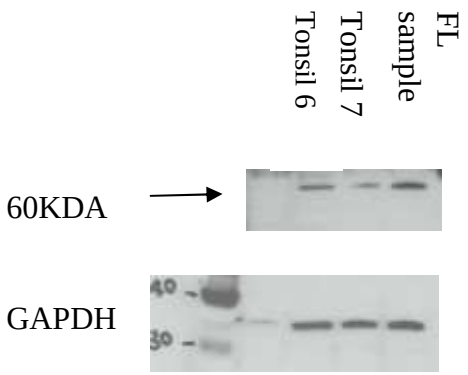
Table 35 Table to demonstrate correlation between the integrity of the FDC meshwork assessed by immunocytochemistry for four antigens on FDC and number of CD68 positive macrophages present

Stain/pattern	**Macrophage number <15/hpf**	**Macrophage number (>15/hpf)**	**P-value**
CD21 Intact	20 (33%)	12 (50%)	0.1
Disrupted	41 (67%)	12 (50%)	
CD23 Intact	35 (59%)	11 (46%)	0.2
Disrupted	24 (41%)	13 (54%)	
CD35 Intact	7 (14%)	3 (16%)	0.6
Disrupted	42 (86%)	16 (84%)	
Clusterin Intact	6 (10%)	5 (25%)	0.1
Disrupted	53 (90%)	15 (75%)	

9.3.2.8 Soluble clusterin is increased in FL samples as compared to tonsil

Western blotting of a two tonsil controls and a single sample of FL cell lysate for clusterin demonstrated an increased band in the FL lane as compared to the tonsils at a level of 60KDa. This is the level for the Clusterin precursor protein and this has been demonstrated to be present at higher levels in tumour tissue as compared to healthy tissue (Pucci S et al. 2004). The amounts of GAPDH were the same for all wells indicating equal loading of the wells with protein lysate.

Figure 58 Western blotting of two tonsil protein lysates and a FL protein lysate using anti-human rabbit polyclonalClusterin-α/β antibody demonstrating an increased band in the FL sample at the level of 60KDa.



9.3.3 The presence of an intact FDC meshwork can predict time to transformation in initial diagnostic biopsies of FL

9.3.3.1 Patient characteristics

Fifty one patients with diagnostic FL biopsies were eligible to be included within this study. The histology was reviewed in all cases. The characteristics of the patient population are listed in **Table 36**. Patients who underwent transformation of FL to DLBCL within three years of diagnosis, the rapidly transforming group (n=26), had a median time to transformation of 1.14 years (range 0-2.99). Patients who transformed eight years or more after diagnosis, the slow transforming group (n=25), had a median time to transformation of 19.02 years (range 8.15-34.02). This difference was significant (p=0.0001). The members of the rapidly transforming were older than the slowly transforming, with median ages at diagnosis of 61 years and 47 years respectively however this was not statistically significant (p=0.18).

Table 36 Demographic and Clinical Characteristics of Patients from the Rapidly Transforming and Slow Transforming Groups

	Rapidly transforming group	**Slow transforming group**
Total no. of patients (n=51)	26	25
Sex		
Male	18	15
Female	8	10
Time to transformation (years)		
Median	1.14	19.02
Range	0 – 2.99	8.15 – 34.02
Age at diagnosis (years)		
Median	61.04	47
Range	30.8 - 72.6	30.5 - 65
Grade	Grade 1 = 13 Grade 2 = 11 Grade 3a = 2	Grade 1 = 14 Grade 2 = 8 Grade 3a = 3
Stage	2 unknown	
I	0	1
II	5	6
III	8	6
IV	11	12
Number of repeat biopsies		15 cases: 1-6 relapses, 1-7 biopsies 6 cases: no relapse, no biopsies 1 case: no relapse, 3 biopsies 3 cases: 1 relapse, no biopsies

9.3.3.2 Disruption of FDC meshwork assessed by immunocytochemistry for CD21 can predict rapid transformation of FL to DLBCL

Analysis of the FDC meshwork using immunocytochemistry for CD21 and CD23 was performed according to the scoring system described in the Materials and Methods. The markers CD35, Clusterin and CNA.42 were not used as this work was performed chronologically prior to the work described earlier in this chapter. The presence of an intact FDC meshwork as assessed by CD21 immunocytochemistry was significantly associated with rapid transformation from FL to DLBCL whereas the presence of a disrupted FDC meshwork as assessed by CD21 immunocytochemistry was significantly associated with slow transformation from FL to DLBCL ($p = 0.0025$). Disruption of the FDC meshwork as analysed by CD23 immunocytochemistry was not associated with any differences in time to transformation ($p=0.4$) (**Table 37**).

Table 37 Correlation of speed of transformation with integrity of the FDC meshwork as assessed by immunocytochemistry for CD21 and CD23

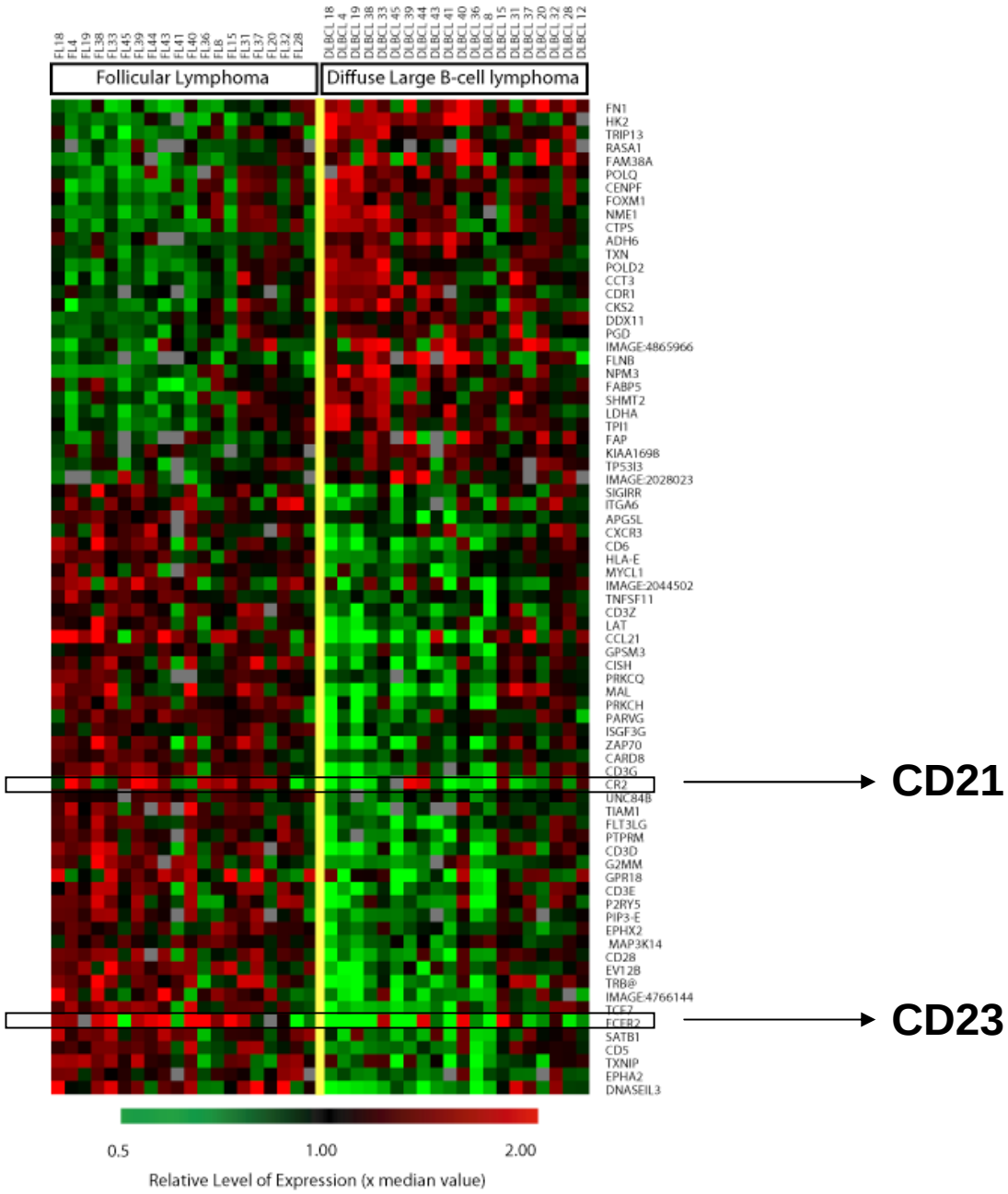
Antigen	**Pattern**	**Fast transforming group**	**Slow-transforming group**
CD21	Disrupted	6/23 (26%)	17/24 (71%)
	Non-disrupted	17/23 (74%)	7/24 (29%)
CD23	Disrupted	7/24 (29%)	5/23 (22%)
	Non-disrupted	17/24 (71%)	18/23 (78%)

9.3.4 Follicular dendritic cell markers are lost on transformation

9.3.4.1 Gene expression profiling of pre- and post-transformation sample of FL

As previously indicated, transformation of follicular lymphoma to an aggressive high grade lymphoma is a common and adverse prognostic event. The gene expression data described in Chapter 4 was obtained from lymph node samples of follicular lymphoma (FL) which subsequently transformed to diffuse large B cell lymphoma (DLBCL) using the Lymphochip cDNA microarray. A list of seventy-six genes whose expression was significantly altered on transformation was generated using the random variance model *t*-test. The cut off value for *P* was set at <0.001. Twenty nine genes demonstrated up-regulation and 47 genes are down-regulated upon transformation (Davies AJ et al. 2006). In order to assess concordance between mRNA and protein expression gene products were chosen for further study using immunocytochemistry. The follicular dendritic cell antigens *CR2 (CD21)* and *FCER2 (CD23)* were the most significantly down-regulated genes on transformation of FL to DLBCL (**Figure 59**) and their protein expression in tumour samples was analysed. Therefore, the protein expression of these genes was analysed on an independent validation set of 34 patients with at least one pre-transformation and one post-transformation paraffin embedded biopsy.

Figure 59 A selection of genes with significant differential expression on transformation of follicular lymphoma to diffuse large B-cell lymphoma (20 paired samples) is shown on the right hand side. Green represents downregulated and red upregulated. Arrows indicate genes chosen for this project. Original image by Dr A J Davies with permission.



9.3.4.2 Loss of FDC markers CD21 and CD23 on transformation of FL to DLBCL confirmed by immunocytochemistry on transformation TMA

The gene expression data has been validated at a protein level on TMA using immunocytochemistry. Samples comprising both FL and t-FL (DLBCL) were available to evaluate expression of both markers in 28 patients. For each antibody, 2 (maximum of 10% cases over all the antibodies studied) or 3 replicate cores were assessable. The CD21 positive FDC meshwork was lost in 15/28 (54%) of patients upon transformation however results demonstrated a clear FDC meshwork in the remaining cases of tFL (**Figure 60**). Similarly CD23 marked the FDC meshwork but was present occasionally on tumour cells. The presence of the FDC meshwork highlighted by CD23 expression was lost in 17/28 (61%) of patients on transformation. The remainder of the cases retained an FDC meshwork on transformation of FL to DLBCL (**Figure 61**). The concordance between the loss of both markers was 71%. There was no correlation with overall survival or survival from transformation for either CD21 (**Figures 62, 63**) or CD23 (**Figures 64, 65**).

Figure 60 Immunocytochemistry for CD21 on paired FL and transformed FL (DLBCL) on TMA demonstrating, A) Well formed discrete meshworks of FDC in FL OM x10, B) DLBCL transformed from FL demonstrating loss of FDC meshwork OM x10.

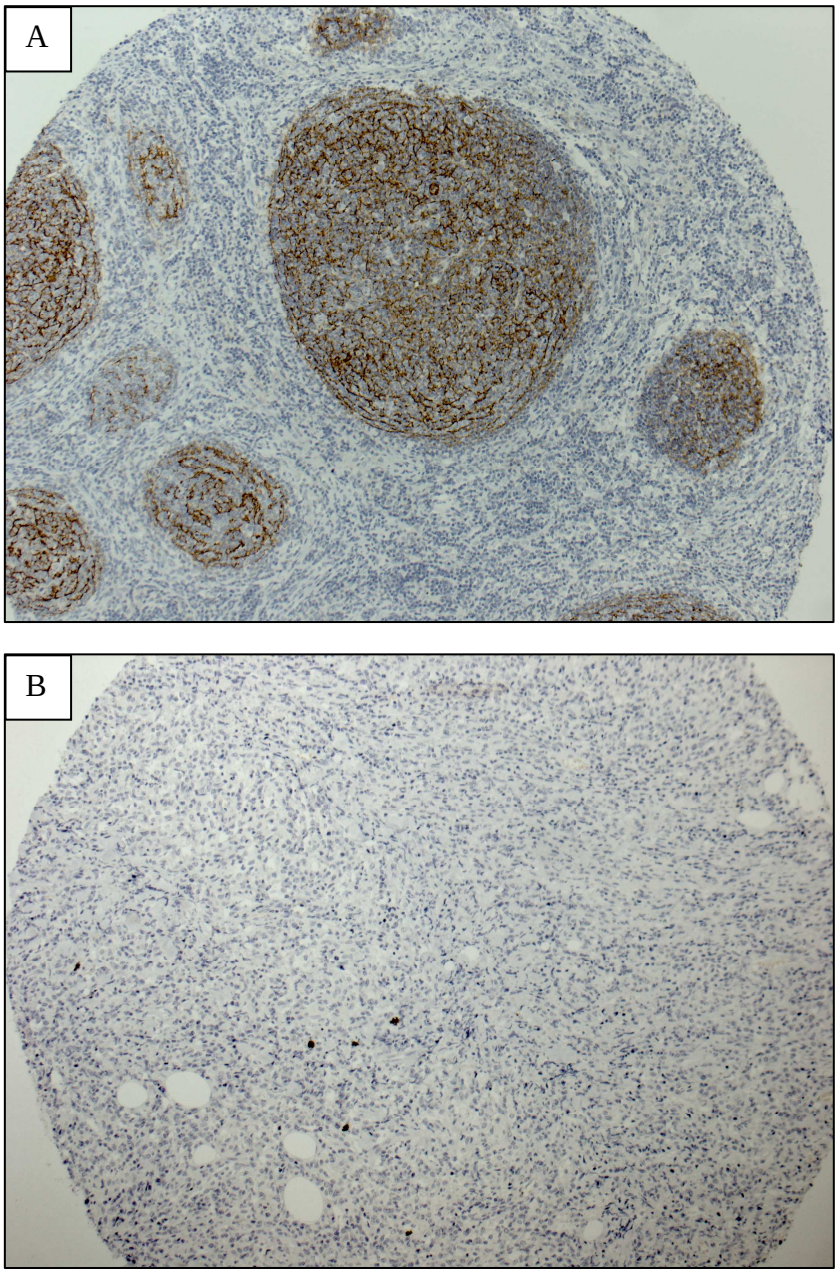


Figure 61 Immunocytochemistry for CD21 demonstrating, A) A neoplastic follicle of FL with a well developed and demarcated FDC meshwork original magnification x20, B) DLBCL transformed from FL demonstrating retention of FDC meshwork within the tumour. Original magnification x20.

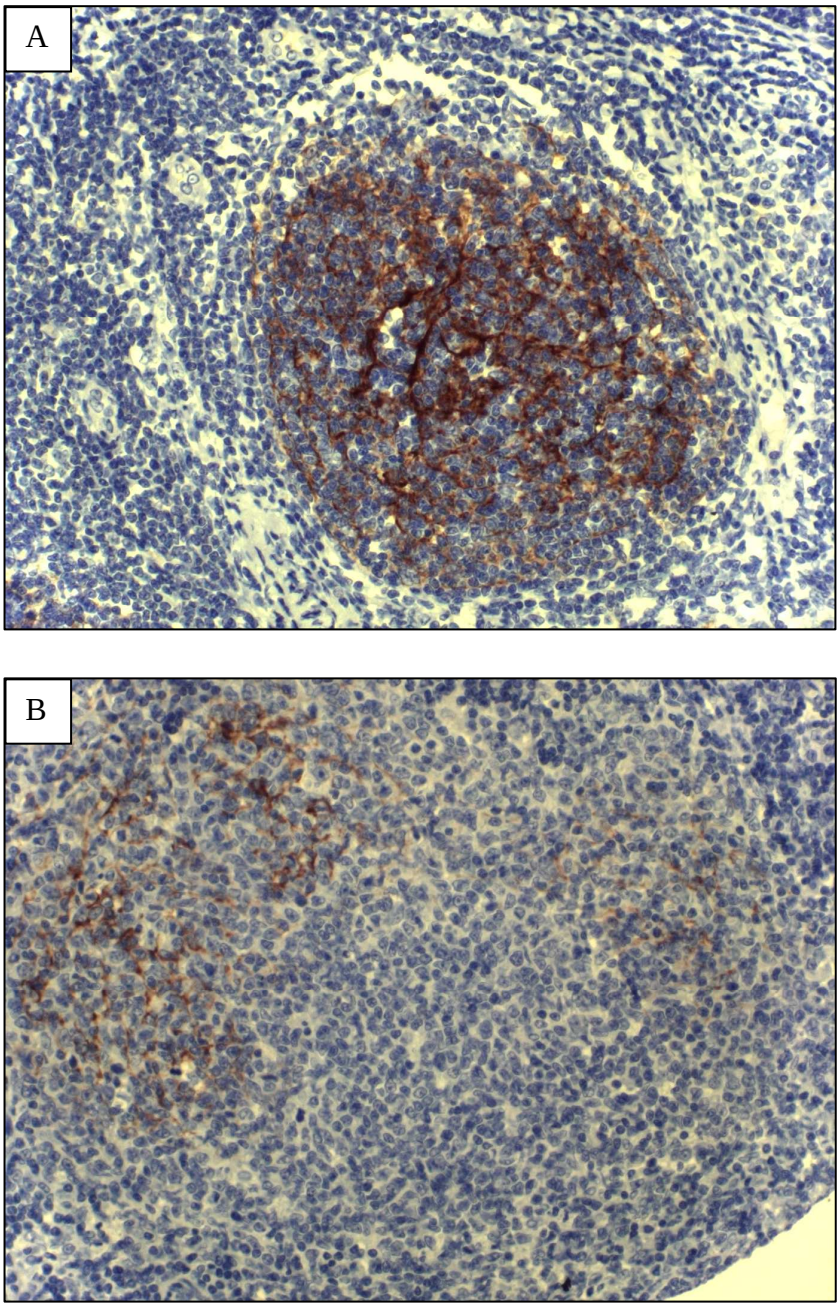


Figure 62 Kaplan-Meier estimate of survival from transformation by expression of CD21 protein analysed by immunocytochemistry (p=0.80). The blue line represents cases where the expression of CD21 by FDC was maintained on transformation of FL to DLBCL. The red line represents cases where the expression of CD21 was lost on transformation of FL to DLBCL

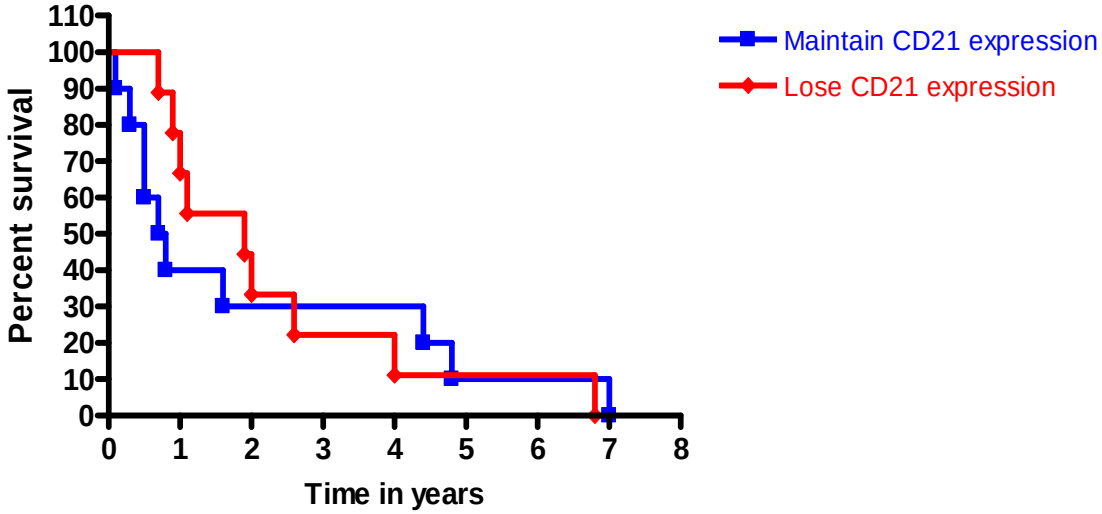


Figure 63 Kaplan-Meier estimate of overall survival by expression of CD21 on transformation of FL to DLBCL. The blue line represents cases where the expression of CD21 by FDC was maintained on transformation of FL to DLBCL. The red line represents cases where the expression of CD21 was lost on transformation of FL to DLBCL (p=0.4)

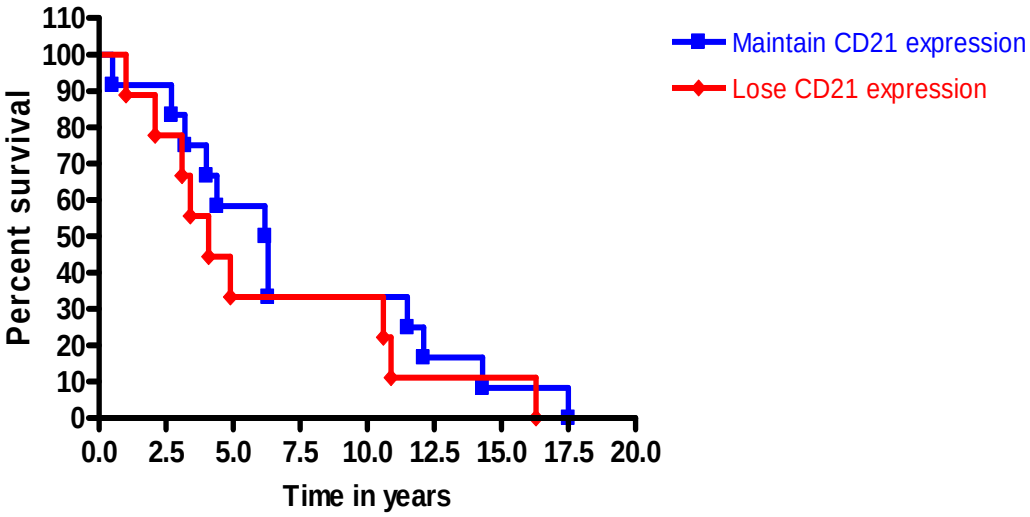


Figure 64 Kaplan-Meier estimate of survival from transformation by expression of CD23 protein on FDC analysed by immunocytochemistry (p=0.9). The blue line represents cases where the expression of CD23 by FDC was maintained or increased on transformation of FL to DLBCL. The red line represents cases where the expression of CD23 on FDC was lost on transformation of FL to DLBCL

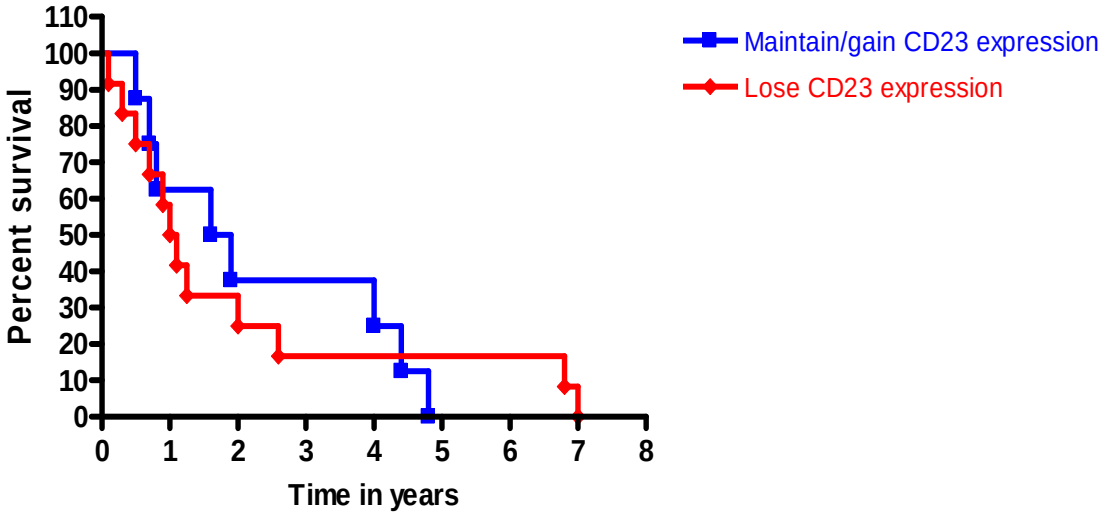
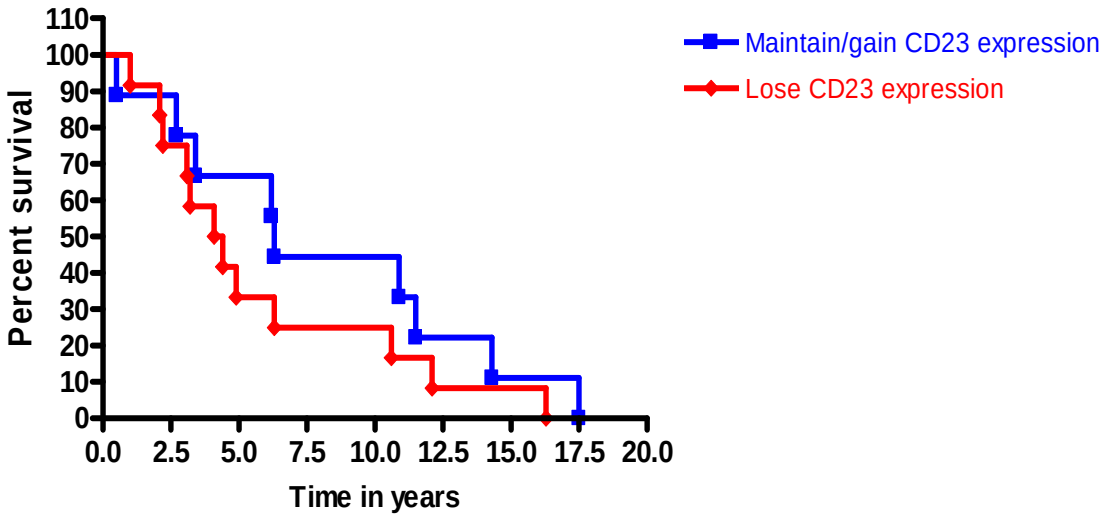


Figure 65 Kaplan-Meier estimate of overall survival by expression of CD23 protein by immunocytochemistry (p=0.3). The blue line represents cases where the expression of CD23 by FDC was maintained or increased on transformation of FL to DLBCL. The red line represents cases where the expression of CD23 by FDC was lost on transformation of FL to DLBCL.



9.4 Discussion

The Follicular Dendritic Cell is an enigmatic cell which is difficult to study. The cells are fragile and are closely apposed to B lymphocytes within the germinal centre making analysis of these cells problematic. In addition, there are few cell lines which are truly representative of the FDC. The important role of FDC in germinal centre formation and the maturation and survival of B lymphocytes is well recognized (Liu YJ et al 1991; Kosco MH, Pflugfelder E, Gray D 1992) and there is evidence that FDC are important in lymphomageneis (Petrasch S et al. 1992). This coupled with the observation that FDC are present within FL even when the neoplasm is present at sites where reactive lymphoid tissue is not normally seen suggest that FDC are important in the biology and possibly the survival of FL. This work has examined the FDC and their immunophenotype in FL biopsies and correlated the expression of markers with overall survival and transformation.

The FDC meshwork has been demonstrated to differ in secondary follicles in reactive lymph nodes compared to neoplastic follicles in FL lymph nodes. There is loss of the polarity of FDC meshwork observed in secondary follicles on analysis of CD23 immunocytochemistry in neoplastic follicles of FL and a consequent increase in colocalization of CD21 and CD23 expression within neoplastic follicles as compared to secondary follicles in reactive lymph nodes. This demonstrates that the FDC meshwork is changed in FL as compared to reactive lymph nodes.

Analysis of the immunocytochemical staining was performed in 94 diagnostic biopsies from patients with FL to analyse whether any markers could be used as prognostic markers to inform treatment at diagnosis. The presence of an intact or disrupted FDC mesh assessed by immunocytochemistry for CD21, CD23 or CD35 was not associated with overall survival or transformation. The presence of an intact FDC meshwork as assessed by immunocytochemistry for Clusterin was significantly associated with increased time to transformation but not overall survival with patients with an intact meshwork as indicated by Clusterin immunocytochemistry more likely to transform. These results indicate that Clusterin present on FDC cells is important in the biology of FL cells as the presence of Clusterin indicates a cohort of patients who are more likely to transform to DLBCL. This result suggests a more widespread role for the use of Clusterin immunocytochemistry in diagnostic biopsies

of FL to try and provide prognostic information to clinicans and aid treatment selection. The lack of association between the integrity of the FDC meshwork as assessed using immunocytochemistry for the other FDC markers CD21, CD23 and CD35 and overall survival indicate that the results are not conclusive and require further investigation. In addition, the median survival of the patient cohort examined is below the median which would be expected for this disease suggesting that by chance a subset of patients with worse prognosis have been selected for investigation. Analysis of a group of patients with a higher median survival may highlight a survival advantage/disadvantage. The other conclusion is that FDC expressing these markers are not important for FL survival.

Preliminary results indicate that pre-soluble clusterin, a precursor of secretory clusterin which is antiapoptotic, is increased in FL samples as compared to tonsil. This result and the presence of an intact FDC meshwork being associated with increased likelihood of transformation may be linked. If the main location of clusterin within the neoplastic follicle is on the FDC meshwork as described in this work the presence of an intact meshwork may provide a scaffold for increased soluble levels of clusterin which could prevent neoplastic cells undergoing apoptosis. The survival of the neoplastic cells could allow accumulation of mutations which push the tumour from a FL phenotype towards a DLBCL phenotype. The presence of FDC have been demonstrated to protect B lymphocytes from chemotherapeutic agents in murine studies (Schwarz YX et al. 1999) and the presence of a well developed FDC meshwork may represent a mechanism of drug resistance for the FL which could result in reduced overall survival and faster transformation. The overexpression of soluble clusterin may provide a therapeutic target for the treatment of FL.

Histopathologists have long observed the loss of follicular dendritic cell meshwork, as indicated by immunocytochemical staining for CD21 and CD23, on transformation of FL to DLBCL. These observations are confirmed both by gene expression profiling which shows reduction in transcripts for these markers and by the immunocytochemical results of this study. CD21 and CD23 are strongly expressed by follicular dendritic cells within normal germinal centres and are required for survival of the germinal centre. Although used as an indicator of tumour

progression (Chang KC et al 2003) there is no clear indication as to whether loss of these antigens results in tumour progression from low grade FL to the more aggressive DLBCL or whether this is a marker to indicate transformation has occurred. This study cannot answer this question. The lack of significant correlation between loss or retention of expression of both CD21 and CD23 and survival could suggest that it is merely a surrogate marker and not biologically important. However, this may simply be a reflection of the relatively small sample size analysed. Analysis of cases (n=15) of FL demonstrated a stable expression of CD21 and CD23 over time in 7 patients (median time between biopsies 2.2 years); a loss of CD21 and CD23 expression over time in 7 patients (median time between biopsies 3.1) and an increase in expression in a single patient (Chang KC et al. 2003). Transformation to DLBCL was not assessed. CD23 expression was lost at an earlier stage than expression of CD21 (Chang KC et al 2003). This suggests that loss of FDC meshwork is an important factor in a subset of patients. The important architectural role of the FDC meshwork in the germinal centre could explain why the loss of the meshwork may be a precipitating factor in transformation of FL to DLBCL. However, if the presence of an intact FDC meshwork, as assessed by CD21 immunocytochemistry, is associated with worse outcome and rapid transformation to DLBCL as described above the presence of the FDC meshwork may aid transformation of FL to DLBCL. The meshwork may provide biological factors which prevent apoptosis and enhance survival as well as providing protection against chemotherapeutic agents which allow tumour cells to acquire mutations which give them a selective advantage.

FDC have been demonstrated to produce and secrete CXCL13 (Ansel KM et al. 2000) which binds to CXCR5, expressed on mature B lymphocytes and a subpopulation of T-helper memory cells (Dobner T et al. 1992). FL cells also express CXCR5 and migrate in response to CXCL13 (Husson H et al. 2002). FDC also produce monocyte chemoattractant protein-1 (MCP-1) which may also recruit FL cells (Husson H et al. 2001). This suggests that FDC may play a crucial role in the recruitment of FL to sites within the body and raises the questions as to whether treatment with targeted therapy against the FDC, in combination with other therapy targeting the neoplastic cells, could be effective in the treatment of FL. The secretion of these factors may also be responsible for the varied immune cells infiltrates

present in the FL microenvironment. The presence of an intact FDC meshwork may provide an increased stimulus for the recruitment of macrophages, a known adverse prognostic factor (Farihna P et al. 2005), to the neoplastic follicles via MCP-1. This again supports the idea that targeted treatment against the FDC, in combination with treatment against the neoplastic cells, could prove an increasingly effective treatment of FL.

CHAPTER TEN:

Discussion

Follicular lymphoma is a disease characterised by numerous relapses and remissions and a median survival of 8-10 years. In addition, a large number of potential therapeutic interventions are available ranging from a simple 'watch and wait' strategy through more aggressive treatments depending upon the requirements of individual patients. Although the median survival is approximately 8-10 years from diagnosis there is a great deal of variability with some patients dying of their disease very rapidly (<5 years from diagnosis) and other patients surviving over thirty years from initial diagnosis. At the present time there is no consensus on which treatments should be used to treat FL (Solal-Celigny P et al. 2004; Gribben J 2007) and predicting patients whose disease will behave in an aggressive manner and so may require intensive therapy is difficult. The FLIPI uses clinical parameters to stratify patients and provides a consistent method to compare clinical trials and evaluate treatments (Solal-Celigny P et al. 2004) however the FLIPI has only limited discriminative power and is rarely used in the clinical setting to drive treatment choices. It has been shown that in patients with FL at first relapse the FLIPI can be used with the presence of B symptoms to predict five-year survival from progression (Montoto S et al. 2004). Between 25-35% of patients with FL transform to DLBCL (Acker B et al. 1983; Gallagher CJ et al. 1986; Montoto S et al. 2007) although post-mortem data suggests a much higher rate of transformation; 68% (Garvin AJ et al. 1983). Transformation is typically a clinically catastrophic event with death often occurring quickly (Bastion Y et al. 1997).

There is a need to identify prognostic biomarkers which can reliably predict the likelihood of a patient having an aggressive disease and the risk of transformation at diagnosis. This would allow stratification of patients into risk groups with appropriate treatment options, preventing the over-treatment of those patients with truly 'indolent' disease. In addition, there is little information about the biology of transformation. Transformation is a complicated process which probably can occur via many pathways, investigation into these mechanisms is important in predicting those patients who will transform as well as identifying putative therapeutic targets.
The inference that the immune microenvironment is important in the biology of FL is not a novel idea; the presence of CD4 T lymphocytes was observed within FL in the 1980's (Swerdlow SH et al. 1985) and their importance was implied by the observation that significantly more CD4 T lymphocytes were present in the biopsies

of untreated FL patients who underwent spontaneous regression (Strickler JG et al. 1988). Gene expression profiling of FL biopsies from untreated patients produced two gene signatures which allowed the grouping of patients into quartiles with difference in survival. The gene signatures were as a result of gene expression of tumour infiltrating cells and were independent of clinical parameters (Dave SS et al. 2004). Corroboration of the findings at a gene expression level were provided by Farinha et al. who demonstrated a correlation between increased numbers of CD68 positive macrophages and poor prognosis although the presence of T lymphocytes did not identify an improved prognosis (Farinha P et al. 2005).

The work outlined in this thesis has validated tissue microarray as a technique for a high throughput approach to the assessment of immunocytochemical markers in FL. Prior to this work a single study had used TMA to analyse a series of 193 B cell NHLs and 29 cases of Hodgkins lymphoma and compared full section staining results to those obtained using TMA and found a high correlation (Hedvat CV et al. 2002). This work demonstrated concordance between scoring immunocytochemistry from TMA cores and full sections of more than 90%. This has verified the technique as an appropriate method for analysis of haematological malignancies. This has been further corroborated by subsequent studies in haematological malignancies (Garcia JF et al. 2003; Tzankov A et al. 2003; Zettl A et al. 2003).

The work reported here has examined the role of the microenvironment in predicting outcome of patients with FL including the likelihood of transformation. A study of 59 diagnostic samples obtained from patients with poor survival (<5 years from diagnosis) and good survival (>15 years from diagnosis) identified CD4 positive T lymphocytes and a perifollicular pattern of FOXP3-positive cells were more commonly found in diagnostic lymph nodes from patients with favourable outcome. These observations are in keeping with the hypothesis that the presence of CD4 positive T lymphocytes within the tumour are of benefit to the host (Strickler JG et al. 1988) and are consistent with the gene expression data (Dave SS et al. 2004). We found no correlation between survival and the presence or location of CD68 positive macrophages, in contrast to a previous report (Farinha P et al. 2005). This may be a reflection of the different patient populations studied since the patients studied here were older and had less aggressive disease than those of Farinha et al (Farinha P et

al. 2005). However, our results are in keeping with the gene expression profiling data in that CD68 was not one of the 24 genes identified by Dave et al (Dave SS et al. 2004) and other genes from that signature should be assessed.

The presence of peri-follicular FOXP3 positive cells was observed in samples from patients with longer survival. This implied regulatory T lymphocytes were important in the biology of FL. Similar results were published by Carreras et al. who observed a high number of FOXP3-positive cells correlated with improved overall survival in FL although no association with location was observed (Carreras J et al. 2006). The association of improved survival and the presence of FOXP3-positive cells is the opposite of that observed in epithelial malignancies where their presence seems to promote tumour tolerance (Curiel TJ et al. 2004). This could be explained by the fact that activated CD4+CD25+ T lymphocytes can kill B lymphocytes using perforin and granzyme (Zhao DM et al. 2006) or that they can lyse antigen presenting B cells by Fas-Fas ligand interaction (Janssens W et al. 2003). In addition regulatory T cells can suppress immunoglobulin production and class switch recombination (Lim HW et al. 2005). All these actions could allow regulatory T cells to disrupt survival and expansion of neoplastic FL cells. The importance of the immune microenvironment on prognosis in FL has been further demonstrated by the observation of differing impact dependent upon different treatment protocols. The presence of a high number of FOXP3 positive cells, numerous macrophages in an interfollicular pattern and preserved FDC meshworks was associated with an improved time to progression in patients treated with CVP, in patients treated with fludarabine this microenvironment was a poor prognostic feature (de Jong D et al. 2009). This treatment effect may explain some of the conflicting evidence currently published with regard to the impact of the immune microenvironment in FL and prognosis.

Although our own findings and those of Farinha are indicative that high numbers of macrophages are important in the outcome of FL, the role macrophages play in the setting of FL is not fully understood. Macrophages migrate into the tumour microenvironment as a result of cytokines produced by tumour cells and MCP-1 produced by FDC (Husson H et al. 2001). Macrophages are polarised into either M1 or M2. M1 is activated in response to microbial products and cytokines (Adams DO, Hamilton TA 1984) and M2 is induced by IL-4 (Stein M et al. 1992), IL13 and IL-10

(Goerdt S, Orfanos CE 1999). The M2 macrophage group are responsible for remodelling and healing and the exposure of macrophages to IL-4 inhibits the phagocytosis of immune complexes and superoxide production (Becker S, Daniel EG 1990). The presence of increased numbers of M2 type macrophages which act to reduce inflammatory reaction against tumour cells could explain the observations of Farinha et al. and this work.

The work in this thesis has also examined the changes in the immune microenvironment on transformation of FL to DLBCL. The observation that the numbers of CD68 positive macrophages was high in all samples except one of FL prior to transformation and remained so on transformation is consistent with the hypothesis proposed by Farinha et al. who observed that short survival from diagnosis of FL correlated with >15 positive cells /hpf in diagnostic biopsies (Farinha P et al. 2005). Transformation of FL to DLBCL is a clinically catastrophic event associated with reduced survival and the results in this study would be in keeping with those of Farinha et al. In contrast to the work of Carreras et al. there was no observed decrease in the number of FOXP3-positive cells on transformation in this work which could as a result of the differing methods used to count the FOXP3-positive cells as well as sample variance (Carreras J et al. 2006). The median overall survival for patients in whose biopsies the number of FOXP3-positive cells decreased on transformation was 10.9 years compared to 3.9 years for those patients whose biopsies contained increased numbers of FOXP3 positive cells on transformation. This result appears different to the results previously observed in this work; a perifollicular location of FOXP3-positive cells was more commonly found in diagnostic lymph nodes from patients with favourable outcome. There are differences in assessment of outcome, as in this part of the study all the patients transformed from FL to DLBCL and in the previous work the overall survival was the outcome factor being assessed. The presence of FOXP-3 positive cells in a perifollicular location in patients with favourable outcome could be explained by the ability of regulatory T cells to kill (Zhao DM et al. 2006; Janssens W et al. 2003) or inhibit B lymphocytes (Lim HW et al. 2005). This could be an effective means of inhibiting tumour growth in a relatively slow growing tumour such as FL. In a rapidly growing tumour such as DLBCL these mechanism may be unable to control tumour growth and may worsen the situation by inhibiting the immune system which

may be trying to produce an anti-tumour effect. This hypothesis could be supported by the observation of high numbers of CD4 and CD8 positive lymphocytes in the tumour samples both pre- and post-transformation. The observation that the immune microenvironment did not impact on outcome in *de novo* DLBCL against supports this hypothesis.

Patients with initial diagnostic biopsies of FL who subsequently transformed to DLBCL were identified and two cohorts of patients were then established. The first cohort of 26 patients transformed to DLBCL in less than 3 years from initial diagnosis of FL and the second cohort of 25 patients transformed after eight years from initial diagnosis. Whilst the absolute numbers of CD4 positive lymphocytes did not correlate with time to transformation the location of these lymphocytes was important. Patients from the rapidly transforming group had CD4 positive cells at a higher incidence within follicles whereas the distribution of CD4 positive lymphocytes was predominantly perifollicular in patients in the slow transforming group. The data suggesting that FL resembling non-malignant hyperplasia including the presence of intrafollicular CD4 positive lymphocytes was associated with rapid transformation support those of a recently published study (Glas AM et al. 2007). Increased numbers of CD68 positive macrophages were more commonly found in the diagnostic FL lymph nodes of patients who underwent rapid transformation to DLBCL. This finding is in keeping with the hypothesis that the lymphoma-associated macrophage content is an independent predictor of survival in FL (Farinha P et al. 2005).

In addition to the impact of the immune microenvironment this work has also analysed some of the molecular mechanisms which might be important in transformation of FL to DLBCL. Currently the molecular mechanisms underlying transformation are poorly understood with numerous proposed mechanisms including mutation of *TP53* (Lo Coco F et al. 1993; Sander CA et al. 1993; Davies AJ et al. 2005); cytogenetic abnormalities (Goff LK et al. 2000; Hough RE et al. 2001); amplifications of *c-REL* (Goff LK et al. 2000); deletions of *CDKN2A* and *CDKN2B* (Elenitoba-Johnson KS et al. 1998); mutations of *Bcl-2* (Matolcsy A et al. 1996) and mutations of *Bcl-6* (Lossos T, Levy R 2000). Each of these has been identified in relatively small numbers of patients and further investigations into the

biological mechanisms are required if treatments and survival for the patients who undergo transformation.

A cohort of twenty nine patients diagnosed with FL which subsequently transformed to DLBCL with both fresh frozen and paraffin embedded pre-transformation and transformed biopsies were identified. The role of *TP53* mutation in transformation was analysed in this patient cohort using a combination of mutation analysis and immunocytochemistry for p53, MDM2 and p21. In the initial diagnostic biopsies of FL no mutations in *TP53* were observed and the percent of patients who acquired mutations on recurrence of lymphoma was low (28%). There were three patients with a FL sample containing a *TP53* mutation prior to transformation. The time period between acquisition of the mutation and transformation to aggressive disease in these patients was variable but in two patients transformation did not occur for 2.5 years and 4 years respectively. The presence of *TP53* mutation is therefore not an indicator of imminent transformation. In addition, the presence or absence of a *TP53* mutation was not associated with survival. This work has also demonstrated that the detection of p53 protein over-expression using immunocytochemistry is not dependent upon *TP53* gene mutation and that the absence of protein does not exclude mutation. There was increased expression of Mdm2 in a high proportion of cases independent of *TP53* mutation suggesting a different mechanism of transformation. The use of small-molecule inhibitors of Mdm2, the Nutlins, may be an option in patients who transform to DLBCL. The nutlins bind to Mdm2 in the p53 binding pocket and their action increases wild-type p53 with subsequent increase in the levels of Mdm2 and p21 suggesting that an intact p53 pathway is required for these drugs to work (Vassilev LT et al. 2004). Initial results in haematological malignancies of treatment with nutlins are encouraging (Kojima K et al. 2005; Stühmer T et al. 2005; Coll-Mulet L et al. 2006).

Paired pre-and post-transformation paraffin embedded tissue samples were obtained from a cohort of 43 patients and using immunocytohemistry this work has demonstrated that FL transforms to DLBCL of GC phenotype. It is surprising that the five year survival from the time of transformation in this study is only 38% despite the majority of patients (89%) having a phenotype shown to be favourable in *de novo* DLBCL. This may reflect previous treatment effect or possibly the

acquisition of further mutations which are advantageous for tumour survival. Gene expression data identified two genes of potential therapeutic importance which were up-regulated on transformation. Aurora Kinase B was also up-regulated at a protein level in the same proportion of patients as identified at a gene expression level. This suggests that AURKB could be an effective target for treatment of patients who have transformed and this is further corroborated by the observation that disruption of AURKB function in a t(14;18) DLBCL cell line results in apoptosis (Harrington EA et al. 2004). With the advent of new drugs selectively targeting Aurora Kinase B which demonstrate anti-proliferative effect on tumours this could prove an important target in some patients who transform from FL to DLBCL. The second up-regulated gene was nm23 and there was a high correlation between the increase in gene transcript level and the increase in protein level detected in patient samples using immunocytochemistry. Increased protein levels of nm23 have previously been correlated with poor prognosis in DLBCL (Niitsu N et al. 1999; Niitsu N et al. 2000) and increased tissue levels of nm23 correlated with increased serum levels (Niitsu N et al. 2004). This could provide an effective method of monitoring patients for transformation of FL to DLBCL using only a relatively non-invasive blood test. As nm23 is only elevated in a subset of patients it cannot be the only method of assessment however. The role of nm23 is to provide high energy nucleosides (Parks RE Jr, Agarwal RP 1973) and these results suggest that the high grade DLBCL may be dependent upon the provision of high energy compounds for survival indicating that nm23 may be a potential therapeutic target.

Follicular dendritic cells are enigmatic cells which are essential for the formation, survival (Lindhout E et al. 1993) and normal function of reactive germinal centres (Liu YJ et al 1991; Kosco MH, Pflugfelder E, Gray D 1992). The presence of FDC in FL which has developed at sites where there was previously no lymphoid tissue coupled with the extreme difficulty in culturing FL *in vitro* without stromal cells suggests that FDC are essential for development and survival of FL. This hypothesis is supported by the demonstration that proliferation of NHL lymphocytes *in vitro* was dependent upon FDC interactions (Petrasch S et al. 1992). The work in this thesis has demonstrated that the FDC meshwork is different in secondary follicles in reactive lymph nodes compared to the FDC meshwork in neoplastic follicles in FL. There is a loss of polarization of CD23 expression on the FDC meshwork in

neoplastic follicles with a pattern of expression similar to that observed in primary reactive follicles. In addition, a change in immunophenotype of FDC was observed. Kasajima-Akatsuka and Maeda observed an immunophenotypic difference in developmental stages and functional phases of human FDC. It was observed that CNA.42 was present on precursors of FDC through all stages of differentiation (Kasajima-Akatsuka N, Maeda K 2006). My own observations of reactive lymph nodes confirmed immunocytochemical staining of FDC meshwork with CNA.42. In all but two of the samples of FL analysed there was loss of the FDC mesh assessed using immunocytochemistry for CNA.42 with individual FDC being positive or negative. The majority of cases (60%) were negative for CNA.42. This suggests that there is a biological difference between FDC in reactive follicles compared to FDC in neoplastic follicles. The finding that only 6.4% of samples contained FDC of a mature immunophenotype (CD21, CD23 and CD35 positive) also indicates that there are differences between FDC in reactive follicles and FDC in neoplastic follicles. The importance of FDC in the biology of FL is implied by the finding that the presence of Clusterin on FDC is associated with increased transformation. Currently assessment of clusterin by immunocytochemistry is not routinely performed on FL diagnostic lymph node biopsies, however, these results suggest that its assessment may provide clinicians with some prognostic information to aid treatment choice. The lack of association between the integrity of the FDC meshwork as assessed using immunocytochemistry for the other FDC markers CD21, CD23 and CD35 and overall survival require further investigation. The preliminary results of this study indicate increased levels of a precursor of secretory clusterin, which is anti-apoptotic, in FL compared to tonsils. This factor could protect the neoplastic cells from apoptosis. It has already been demonstrated that FDC can protect B lymphocytes from chemotherapeutic agents in mice (Schwartz YX et al. 1999). FDC also produce CXCL13 (Ansel KM et al. 2000) the receptor for which CXCR5, is expressed by FL cells. FL cells migrate in response to CXCL13 (Husson H et al. 2002). This suggests that FDC may play a crucial role in the recruitment of FL to sites within the body and raises the questions as to whether treatment with targeted therapy against the FDC, in combination with other therapy targeting the neoplastic cells, could be effective in the treatment of FL. FDC also produce monocyte chemoattractant protein-1 (MCP-1) which may also recruit FL cells (Husson H et al. 2001) as well as potentially macrophages which have previously been shown to be an adverse prognostic factor

in FL (Farinha P et al. 2005). The data in this thesis has shown no association between the presence of an intact FDC meshwork and the number of macrophages suggesting that cytokine production by FDC is not the most important factor in macrophage recruitment.

Is it possible to unite the molecular abnormalities present in the neoplastic cells of FL with the impact of the microenvironment into a model for the pathogenesis of follicular lymphoma? The cytogenetic abnormality which is the hallmark for FL is the t(14;18)(q32;q21) which is present in approximately 70-95% of cases (Horsman DE et al. 1995). This results in the *BCL2* gene being juxtaposed to the immunoglogulin heavy chain gene and results in the over-expression of Bcl2. This cytogenetic abnormality is not sufficient for the development of FL and in the majority of cases there are numerous other abnormalities most commonly involving chromosomes 1, 2, 4, 5, 13, and 17 or additions of X, 7, 12, or 18 (Tilly H et al. 1994). The average number of genetic aberrations increases with grade with increased numbers of complex abnormalities in patients with higher grade tumours (Viardot A et al. 2002). Investigation into the temporal relationship of cytogenetic abnormalities demonstrated -6q, der(18(t(14;18) and +7 are early changes (Hoglund M et al. 2004) with adverse prognostic significance associated with abnormality of chromosome region 1p21-22, 6q23-36 and the short arm of chromosome 17 (Tilly H et al. 1994). A dual pathway model has been proposed by de Jong where the initial early event is the apoptotic resistance conveyed by the t(14;18). Secondary cytogenetic alterations result in a division into two prognostic pathways. The poor prognostic pathway typically involves abnormalities such as -6q and +1q and this produces an immune microenvironment with an activated FDC meshwork and T cells, transformation would be an early event in this pathway. The good prognosis pathway typically possess mutations such as +7/+8, +der(18)(Hoglund M et al. 2004). The result is stabilization of the tumour cells in an inactive immunologic context resembling inactive lymphoid tissue. The rate of genomic mutations is low and transformation occurs slowly and the prognosis is good (de Jong D 2005). The findings in my work would certainly support the immunological aspects of this theory, however the current dearth of information from numerous sources some of which conflict, requires multi-institutional studies of large patient numbers to conclusively answer some of the important questions about the impact of the immune

microenvironment. Once validated as prognostically important, the findings can be easily translated to the routine diagnostic histopathology setting and can be used to identify patients with potentially aggressive disease at the time of diagnosis. Only at this point can definitive treatment strategies which target the biology of the disease be developed.

Chapter 11: References

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Fludarabine Plus Mitoxantrone With and Without Rituximab Versus CHOP With and Without Rituximab As Front-Line Treatment for Patients With Follicular Lymphoma. *Journal of Clinical Oncology* 22(13): 2654-2661.

Appendix I: Individual Contributions

This work was performed whilst I was a Clinical Research Fellow in the CRUK Department of Medical Oncology at St Bartholomews Hospital, London.

The patient samples used in Chapters 3, 4 and 5 were identified by myself and Dr AJ Davies. I identified the patients samples used for Chapters 6 to 9 using the database complied by Dr AJ Norton. Clinical data was provided by the data managers from the CRUK Medical Oncology Unit; Finlay MacDougall, Janet Matthews and Andy Wilson. Cryopreserved samples were retrieved from the tissue bank of the Medical Oncology Unit and also the Department of Histopathology. Identification of several sample pairs was assisted by the use of a database created by Dr Kim Last. I retrieved the Histopathology blocks from the archives of St Bartholomew's Hospital.

All the H&E slides were reviewed by myself and marked for TMA construction. TMA construction was performed by myself with assistance from Andrew Clear and Kelly Morris. Block sectioning was perfomed by myself with assistance from Andrew Clear and Kelly Morris. Immunocytochemistry was performed by myself with assistance from Andrew Clear and Kelly Morris.

Optimization of novel antibodies and staining of TMA sections was performed by myself with assistance from Andrew Clear.

Analysis and scoring of full sections and TMA sections was performed by myself and Dr M Calaminici.

I prepared the cells for Flow cytometry, performed the surface and intracellular staining and the Flow cytometry. I also analysed the results.

I performed and analysed the double immunofluorescent staining. I extracted and quantified the protein from the samples and performed the Western blotting.

I performed statistical analyses with help from Finlay MacDougall. The assistance of Sharon Love, CRUK Medical Statistics Group, Oxford and John-Baptiste Crazier

CRUK Bioinformatics and BioStatistics for formal statistical review of Chapter 5 is gratefully acknowledged.

Immunocytochemistry for p53, MDM2 and P21 was optimised and performed by myself and Andrew Clear. Scoring of immunocytochemistry was peformed by myself and Dr M Calaminici. I performed the cell culture, protein extraction from the cells and the Western blotting. *TP53* mutation analysis was performed by Dr AJ Davies with the assistance of Dr Claire Taylor and Dr Darren Cuthbert-Heavans from the Cancer Research UK Mutation Detection Unit, Leeds

Lymphochip gene expression profiling was performed by Dr AJ Davies in the Laboratory of Dr Lou Staudt, National Cancer Institute, Bethesda, USA under the direct supervision of Dr Andreas Rosenwald.

Appendix II: Publications and abstracts arising from this thesis

Davies A.J; Lee A.M.; Taylor C; Clear A; Goff L.K.; Iqbal S; Cuthbert-Heavens D; Calaminici M; Norton A.J; Lister T.A; Fitzgibbon J (2005) A limited role for TP53 mutation in the transformation of follicular lymphoma to diffuse large B-cell lymphoma**. Leukemia**; 19(8):1459-65

Lee AM, Clear A, Calaminici M, Davies AJ, Jordan S, MacDougall F, Matthews J, Norton AJ, Gribben JG, Lister TA, Goff LK (2006) Number of CD4-positive cells and location of both FOXP3-positive and CD25-positive cells in diagnostic follicular lymphoma tissue microarrays correlates with outcome. **Journal of Clinical Oncology;** 24(31): 5052-9

Davies AJ, Rosenwald A, Wright G, Lee A, Last KW, Weisenberger D, Chan WC, Delabie J, Braziel RM, Campo E, Gascoyne RD, Jaffe ES, Muller-Hermelink HK, Ott G, Calaminici M, Norton AJ, Goff LK, Fitzgibbon J, Staudt LM, Lister TA (2006) Transformation of follicular lymphoma to diffuse large B-cell lymphoma proceeds by distinct oncogenis mechanisms **British Journal of Haematology** 136: 286-293.

The Role Of *TP*53 In The Transformation Of Follicular Lymphoma To Diffuse Large B Cell Lymphoma

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POSTER PRESENTATION AT XII Meeting European Association for Haematopathology

September 26th – October 1st, 2004, Makedonia Palace, Thessaloniki, Greece

Tissue Microarray Is A Useful Tool In The Evaluation Of Genes Implicated In Transformation Of Follicular Lymphoma

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POSTER PRESENTATION AT Pathology Society Winter Meeting, London: 6-7 January 2005

Tissue Microarray Is A Useful Tool In The Evaluation Of Genes Implicated In Transformation Of Follicular Lymphoma

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POSTER PRESENTATION AT AMERICAN SOCIETY OF HEMATOLOGY MEETING, SAN DIEGO: 4-7 DECEMBER 2004

Discrimination of Long and Short Survival in Follicular Lymphoma (FL) Patients by Incidence of FOXP3 and Perifollicular Incidence of CD4 and CD7 Using Immunohistochemistry on Tissue Microarray (TMA) of Diagnostic Lymph Nodes (LN).

LK Goff, AM Lee, AJ Clear, M Calaminici, AJ Davies, S Jordan, F MacDougall, J Matthews, AJ Norton, JG Gribben, TA Lister.

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ORAL PRESENTATION AT THE AMERICAN SOCIETY OF HEMATOLOGY MEETING, ATLANTA, USA: 10-13 DECEMBER 2005

Number of CD4-positive cells and location of both FOXP3-positive and CD25-positive cells in diagnostic follicular lymphoma tissue microarrays correlates with outcome.

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